THE EFFECTS OF INCREASED RESPIRATORY MUSCLE WORK ON BIOMARKERS OF MUSCLE DAMAGE

A Thesis submitted by

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(BSc Physiotherapy, M.Phil. Physiology)

For the award of

Doctor of Philosophy

2024

ABSTRACT

The current techniques to measure respiratory muscle damage are invasive and/or costly. A simple and minimally invasive approach to identify the presence of muscle damage that can accommodate the internal location of the respiratory muscles is the use of blood biomarkers. The overall aim of this research was to investigate whether damage to the respiratory muscles occurs after increased respiratory muscle work and to determine if serum biomarkers can be used to measure respiratory muscle damage. Study 1 investigated respiratory muscle damage following inspiratory pressure threshold loading (ITL). Seven healthy men (33 ± 2 years) undertook 60 minutes of ITL at a resistance equivalent to ~0% (Sham ITL) and 70% of their maximal inspiratory mouth pressure (P_{Imax}) two weeks apart. Serum was collected before and at 1, 24, and 48 h after each ITL session. Creatine kinase muscle-type (CKM) was higher at 1 and 24 hours, fast skeletal troponin-I (sTnI) at 1 hour, while slow sTnI was higher at 48 hours post-ITL. Study 2 investigated the effects of volitional hyperpnea (VH) on biomarkers of respiratory muscle damage. Eight healthy men (33 ± 2 years) undertook 10 min of volitional hyperpnea (VH) and rest (control) two weeks apart. VH involved mimicking the breathing and diaphragm recruitment patterns in a square wave manner to a level equivalent to those at 85% of their maximum minute ventilation produced during a maximal incremental cycling test. Serum was collected before and at 1, 24, and 48 hours after both control and VH trials. Only slow sTnI was significantly higher at 24 h post VH as compared to same time point of control trial. Study 3 investigated the effects of inspiratory muscle training (IMT) on muscle damage biomarkers, respiratory function, and functional capacity in coronavirus disease (COVID-19) recovered young adults, successfully weaned from mechanical ventilation. The IMT group performed 30 dynamic inspiratory efforts twice daily at 50% of their P_{Imax} while the Control group performed 60 inspiratory efforts at 10% of P_{Imax} daily. Serum was collected at baseline, week two, and week four. CKM and slow sTnI were lower at two and four weeks for the IMT compared to the control group. This thesis provides evidence that serum CKM and fast sTnI could be used to assess respiratory muscle damage immediately, while CKM and slow sTnI could be used to assess respiratory muscle damage at later stages following conditions or diseases that elevate respiratory muscle work or activity and cause respiratory muscle damage.

CERTIFICATION OF THESIS

I Muneeb Iqbal declare that the PhD Thesis entitled **The effects of increased respiratory muscle work on biomarkers of muscle damage** is not more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references, and footnotes.

This Thesis is the work of Muneeb Iqbal except where otherwise acknowledged, with the majority of the contribution to the papers presented as a Thesis by Publication undertaken by the student. The work is original and has not previously been submitted for any other award, except where acknowledged.

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STATEMENT OF CONTRIBUTION

Paper 1:

Published manuscript: Muneeb Iqbal, Edward Bliss, Eliza Whiteside, Ben Hoffman, Dean E. Mills. (2023). Biomarkers to measure respiratory muscle damage following inspiratory pressure threshold loading in healthy young men. *Journal of Applied Physiology 134:* 1038–1046. https://doi.org/10.1152/japplphysiol.00656.2022

Conference presentation: Respiratory failure and mechanical ventilation conference by European Respiratory Society at Berlin, Germany, held on 9-11 June, 2022.

Published abstract: Muneeb Iqbal, Edward Bliss, Eliza Whiteside, Ben Hoffman, Dean E Mills (2022). Respiratory Muscle Damage Biomarkers in Response to Inspiratory Pressure Threshold Loading in Healthy Young Adults. *European Respiratory Journal Open Research; 8: Suppl. 9,* 11. https://dx.doi.org/10.1183/23120541.Rfmvc-2022.11

Statement of contribution: Muneeb Iqbal contributed 70% to this paper. Edward Bliss, Eliza Whiteside, Ben Hoffman, Dean E. Mills collectively contributed the remainder.

Paper 2:

Submitted manuscript: Muneeb Iqbal, Edward Bliss, Eliza J. Whiteside, Ben Hoffman, Dean E. Mills. The effects of volitional hyperpnea on biomarkers of respiratory muscle damage in healthy young men. Manuscript has been submitted to the Journal of Applied Physiology and is at revision stages.

Statement of contribution: Muneeb Iqbal contributed 70% to this paper. Edward Bliss, Eliza J. Whiteside, Ben Hoffman, Dean E. Mills collectively contributed the remainder.

Paper 3:

Submitted manuscript: Muneeb Iqbal, Kumail Hassan, Eliza J. Whiteside, Ben Hoffman, Dean E. Mills. The effects of inspiratory muscle training on biomarkers of respiratory muscle damage in recovered COVID-19 patients after weaning from mechanical ventilation. Manuscript has been submitted to Chronic Respiratory Disease and is currently under review.

Conference presentation: 47th Annual Meeting of the Austrian Society for Pulmonology at Graz, Austria; held on 23 - 25 October, 2023.

Published abstract: Muneeb Iqbal, Kumail Hassan, Eliza J. Whiteside, Ben Hoffman, Dean E. Mills (2023). *The Central European Journal of Medicine 135:* 548–589. https://doi.org/10.1007/s00508-023-02286-1 (P30).

Statement of contribution: Muneeb Iqbal contributed 70% to this paper. Kumail Hassan, Eliza J. Whiteside, Ben Hoffman, Dean E. Mills collectively contributed the remainder.

ACKNOWLEDGEMENTS

I extend heartfelt thanks to the University of Southern Queensland for providing me with this opportunity and the essential facilities. I wish to acknowledge the collaborative efforts of my supervisory team. The impact of Dean Mills has been transformative, and my gratitude knows no bounds. Dean, you have guided me into the field of research as if holding a child's hand. From practical experimental trials in the lab, through data analysis, write-ups, and up to publication and thesis, you have worked harder than me at every step. Guiding me not only through academic challenges but also through the intricate maze of being an international student during difficult times, your mentorship went beyond scholarly guidance, offering a compassionate hand through personal and global uncertainties. Professor Eliza J. Whiteside, I extend my sincere gratitude for your expert guidance throughout. Your encouragement and support during the times of rejection infused me with hope and enthusiasm. Dr. Ben Hoffman, I appreciate your exceptional and detailed expert reviews on all of my work. Your contributions significantly elevated the quality of my writings and research, Thank you. I would like to mention the wonderful practical support and expert advice on all blood-related matters provided by Dr. Edward Bliss. Appreciation is extended to all those involved during my PhD journey, including my research participants, UniSQ staff and the supportive Graduate Research School team.

Lastly, heartfelt thanks to my parents, siblings, wife, and kids for their ongoing support. To my parents, your prayers have been my guiding light, infusing my journey with strength and resilience. To my life partner, Tanzeela Nisar, I offer profound thanks for firm support, enduring patience, and an unshakeable belief in my capabilities. Love to my kids Hoorain Fatima and Muaz Iqbal; being with you is always a big relief for me from any kind of stress and tough times.

This research has been supported by the Australian Government Research Training Program Scholarship.

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ABBREVIATIONS

6MWT	Six minute walk test
ANOVA	Analysis of variance
AST	Aspartate transaminase
ATP	Adenosine triphosphate
B*f*	Breathing frequency
BMI	Body mass index
CA III	Carbonic anhydrase III
CK	Creatine kinase
CKM	Creatine kinase muscle type
CO_2	Carbon dioxide
CON	Control
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease
cTnI	Cardiac troponin I
DOMS	Delayed onset muscle soreness
ECG	Electrocardiogram
EMG	Electromyography
EMG_{int}	Electromyography of the intercostal
EMG_{para}	Electromyography of the parasternal
EMG_{scm}	Electromyography of the sternocleidomastoid
EELV	End-expiratory lung volume
EILV	End-inspiratory lung volume
ELISA	Enzyme linked immunosorbent assay
FABP3	Fatty acid-binding protein 3
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
HR	Heart rate
IC	Inspiratory capacity
IMT	Inspiratory muscle training
ITL	Inspiratory pressure threshold loading
LDH	Lactate dehydrogenase

O_2	Oxygen
MRI	Magnetic resonance imaging
Myl3	Myosin light chain 3
P_{di}	Transdiaphragmatic pressure
P_{di} Peak	Peak transdiaphragmatic pressure
P_{es}	Esophageal pressure
$P_{ET}CO_2$	Partial pressure of end-tidal carbon dioxide
P_{ga}	Gastric pressure
P_M	Inspiratory mouth pressure
P_{Imax}	Maximal inspiratory mouth pressure
PTP_e	Esophageal pressure time product
PTP_{di}	Transdiaphragmatic pressure time product
QoL	Quality of life
RMS	Root mean squared
RPD	Rating of perceived dyspnea
SaO_2	Estimated arterial oxygen saturation
SD	Standard deviation
sTnI	Skeletal troponin I
TnI	Troponin I
Ti/ToT	Duty cycle
VAS	Visual analogue scale
$\dot{V}_E$	Minute ventilation
$\dot{V}_{Emax}$	Maximum minute ventilation
VH	Volitional hyperpnea
$\dot{V}O_2$	Oxygen uptake
$\dot{V}O_{2max}$	Maximal oxygen uptake
V_T	Tidal volume

CHAPTER 1: INTRODUCTION

Chapter 2 is a literature review evaluating exercise-induced skeletal muscle damage and the available methods to measure this damage, including commonly used and recommended blood biomarkers of skeletal muscle damage. This chapter then reports respiratory muscle work and respiratory muscle damage in various respiratory conditions (i.e., coronavirus disease; COVID-19). The review evaluates potential causes, underlying mechanisms, and what are the problems associated with respiratory muscle damage and why is it important to measure this damage. The review also examines how currently available methods to measure peripheral skeletal muscle damage are not suitable or applicable to measure respiratory muscle damage. The chapter also describes the use of blood biomarkers to measure respiratory muscle damage and interventions to reduce respiratory muscle damage in various respiratory diseases. A summary of this literature review is provided at the end of this chapter, describing the literature gap and the need for the research studies.

Chapter 3 is the first study of the PhD thesis and titled “Biomarkers to measure respiratory muscle damage following inspiratory pressure threshold loading (ITL) in healthy young men”. The chapter describes how detecting respiratory muscle damage induced by whole-body exercise, such as cycling, through the interpretation of blood biomarkers can be challenging due to the unidentifiable site of release. An experimental method that allows isolated exercise of respiratory muscles, excluding peripheral muscle involvement, is ITL. This study used this technique to increase inspiratory muscle work in resting healthy individuals, isolating respiratory muscles and attributing any changes in systemic blood biomarkers to these muscles. A panel of biomarkers including creatine kinase muscle-type (CKM), myoglobin, fatty acid-binding protein-3 (FABP3), myosin light chain-3 (Myl3), and fast and slow skeletal troponin-I (sTnI) were employed to assess respiratory muscle damage in response to ITL undertaken on separate occasions at 70% (high ITL) and ~0% of maximal inspiratory mouth pressure (P_{Imax}) (Sham ITL) in healthy young men. This chapter concluded that CKM and fast sTnI could be used to assess respiratory muscle damage immediately (+1 h), and CKM and slow sTnI could be used to assess respiratory muscle damage 24 and 48 h following conditions that cause elevated inspiratory muscle work.

Chapter 4 describes the second study of the PhD thesis and titled “The effects of volitional hyperpnea (VH) on biomarkers of respiratory muscle damage in healthy young men”. In this study, another experimental approach was used that also allows respiratory muscles to be exercised without peripheral muscle involvement which was VH performed at rest. This is a more ecologically relevant way to test whether respiratory muscle damage occurs following high intensity exercise hyperpnea. The VH challenge mimicked the breathing (tidal volume, breathing frequency and duty cycle) and diaphragm recruitment (transdiaphragmatic pressure) patterns achieved during high intensity exercise. In this chapter, the response of a panel of serum biomarkers, consisting of CKM, and fast and slow sTnI was used to detect the presence of respiratory muscle damage in response to VH and control trials undertaken on separate occasions in healthy young men. This chapter concluded that that only slow sTnI was higher at +24 h post VH compared to the same timepoint after control trial. CKM and fast sTnI did not increase after VH compared with the control trial. These results suggest that respiratory muscle damage was present at +24 h following VH based upon the serum slow sTnI findings, but not evidenced by the serum CKM and fast sTnI findings.

Chapter 5 investigated the effects of inspiratory muscle training (IMT) on biomarkers of muscle damage in recovered COVID-19 patients after weaning from mechanical ventilation. COVID-19 patients experience respiratory muscle damage, leading to reduced respiratory function and functional capacity often requiring mechanical ventilation which further increases susceptibility to muscle weakness. This chapter details that IMT has been found to be beneficial in the recovery from mechanical ventilation-induced respiratory muscle dysfunction and can improve respiratory function and functional capacity by increasing muscle strength and endurance. IMT may help to restore the muscle coordination lost during mechanical ventilation, by training the muscles to work together in a synchronized manner, resulting in a more enhanced respiratory muscle function. This may result in lower concentrations of biomarkers of muscle damage. However, whether IMT could reduce biomarkers of muscle damage and improve respiratory function and functional capacity in recovered COVID-19 patients after weaning from mechanical ventilation was not studied before. This study investigated the effects of IMT on muscle damage biomarkers, respiratory function, and functional capacity in COVID-19 recovered young adults, successfully

weaned from mechanical ventilation. Participants were randomly allocated to either an IMT or control (CON) intervention for four weeks. The IMT group performed 30 dynamic inspiratory efforts twice daily, at 50% of their P_{Imax} while the CON group performed 60 inspiratory efforts at 10% of P_{Imax} daily. Statistical analysis revealed time x group interaction effects for CKM and slow sTnI, but not for fast sTnI. Both were lower at two and four weeks for the IMT compared to the CON group. Time x group interaction effects were observed for forced expiratory volume in one second, forced vital capacity, P_{Imax} and right- and left-hand grip strength. These were higher for the IMT compared to the CON group. In conclusion, four weeks of IMT decreased muscle damage biomarkers and increased respiratory function and grip strength in recovered COVID-19 patients after weaning from mechanical ventilation. This chapter suggested the inclusion of IMT into the management of COVID-19 patients, particularly for intensive care patients, could assist with their recovery.

1.1. Research aims and hypotheses

The overall aim of this research was to investigate whether damage to the respiratory muscles occurs after increased respiratory muscle work and to determine if serum biomarkers can be used to measure respiratory muscle damage. To achieve this, three research studies were undertaken:

Study 1 (Chapter 3): An observational study was conducted to examine changes in respiratory muscle damage biomarkers in response to ITL in healthy young men. Accordingly, the aim of this study was to investigate the response of a panel of biomarkers including fast and slow sTnI, CKM, FABP3, Myl3 and myoglobin in response to ITL undertaken on separate occasions at 70% (high ITL) and ~0% of P_{Imax} (Sham ITL) in healthy young men. The hypothesis was that these biomarkers would increase following the 70% ITL condition compared to the sham ITL condition.

Study 2 (Chapter 4): An observational study was conducted to examine the effects of VH on biomarkers of respiratory muscle damage in healthy young men. Accordingly, the aim of this study was to investigate the effects of VH on biomarkers of respiratory muscle damage including CKM, and fast and slow sTnI. The hypothesis was that CKM, fast and slow sTnI would increase following VH compared to a control trial.

Study 3 (Chapter 5): A randomized control trial was conducted to investigate the effects of IMT on biomarkers of muscle damage in recovered COVID-19 patients after weaning from mechanical ventilation. Accordingly, the aim of this study was to investigate the effects of IMT on biomarkers of muscle damage, respiratory function and functional capacity in recovered COVID-19 patients after weaning from mechanical ventilation. The hypothesis was that IMT would reduce muscle damage and improve respiratory function and functional capacity.

CHAPTER 2: LITERATURE REVIEW

2.1. Exercise-induced skeletal muscle damage

Skeletal muscle damage can be defined according to morphological or physiological indices. More commonly, muscle damage is expressed as decreased force-producing ability due to disruptions in regular myofibrillar structure [1]. Exercise-induced skeletal muscle damage can be defined as structural disruption in the skeletal muscle caused by increased exercise and/or physical activity [2, 3]. The causes are typically excessive, intense or unaccustomed exercise [4]. Following such exercise, there is muscle soreness, decreased pressure pain threshold, localized swelling, and temporary reductions in muscle strength, power and range of motion in the affected limb [4]. This results in various functional impairments depending upon the location of the affected skeletal muscle [5]. Skeletal muscle damage can be particular to just a few macromolecules of muscle tissue, or result in large tears in the sarcolemma [6], z-disk [7], basal lamina [8], and supportive connective tissues [9], and induce damage to the cytoskeleton and contractile elements [10-13]. Muscle injury and exercise-induced muscle damage have been described as a continuum of injury whereby microtears (muscle damage) lead to muscle strain injury [14]. Figure 2.1 demonstrates muscle damage and mechanisms, depicting the structural characteristics of skeletal muscle, showing the temporal aspects of muscle performance following exercise-induced damage, and the potential mechanisms behind exercise-induced muscle injuries.

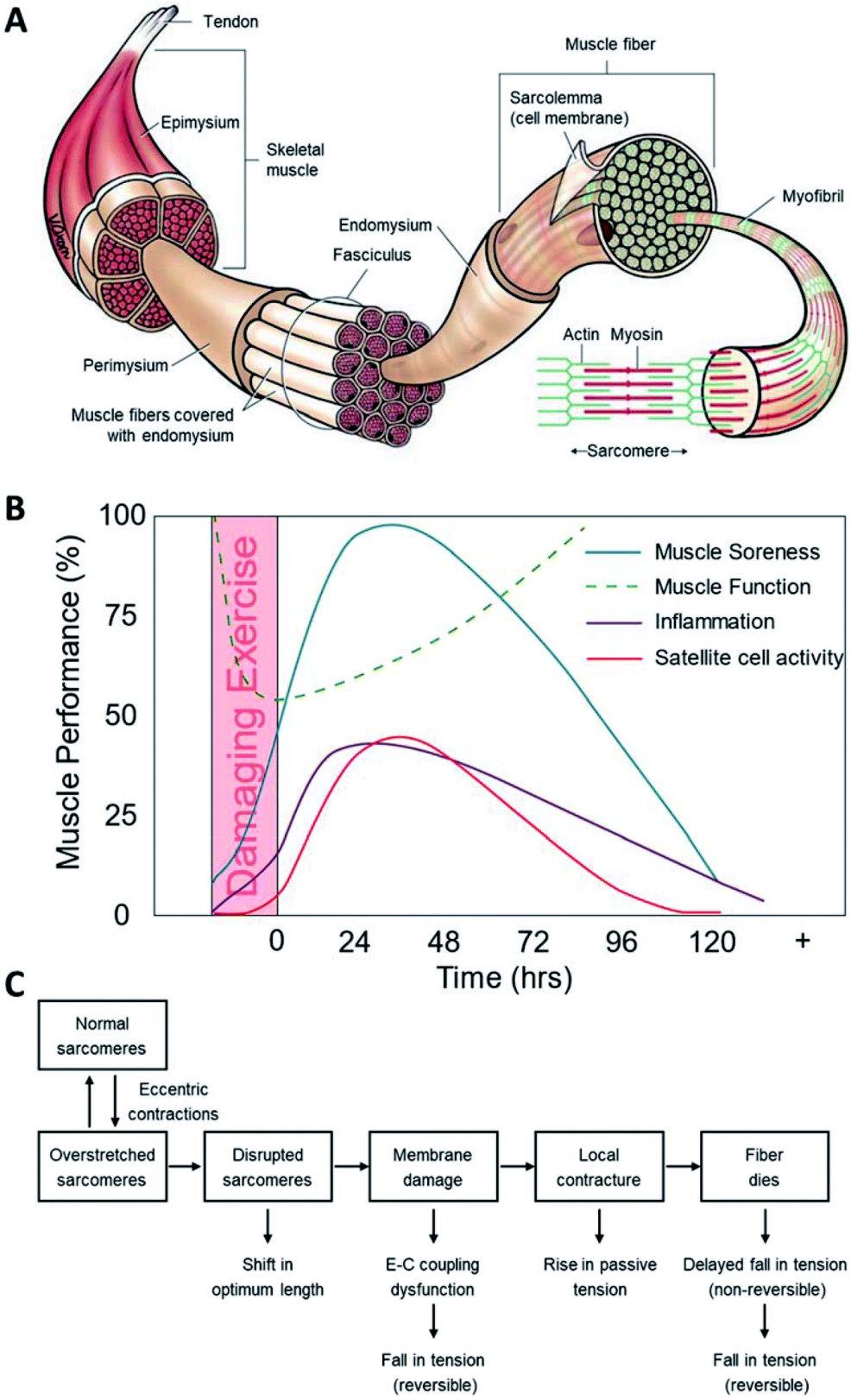

Figure 2.1 Muscle damage and mechanisms. (A) Structure of skeletal muscle; (B) muscle performance over time after exercise-induced muscle damage; and (C) mechanisms of exercise-induced muscle damage and injury [15].

2.2. Measurements of skeletal muscle damage

Multiple methods can be used to measure skeletal muscle damage, and each has limitations. Participant self-reporting of clinical symptoms or history can be ambiguous and subjective in nature with differences in terminology [16]. Muscle biopsies with histopathology would be considered a reference method but are invasive, require skilled technical resources, and are often unnecessary for diagnosis [16]. Several imaging modalities are available including ultrasound, computed tomography scans, magnetic resonance imaging (MRI), magnetic resonance spectroscopy, and positron emission tomography. All of these imaging techniques are costly and outcomes can be technique dependent [17]. Functional assessments such as electromyography (EMG) may be difficult to administer and interpret due to participant cooperation and motivation, and issues with between day reliability [16]. Laboratory diagnostic tests using blood or urine samples are frequently included in myopathy diagnoses. These tests are readily available, provide quantitative, broadly accepted results, and are relatively inexpensive to perform (Table 2.1) [16].

Table 2.1 Biomarkers of skeletal muscle damage.

Biomarker and reference	**Peak increase from baseline**	**Peak time**
Creatine kinase [18, 19]	4-fold	- 8 h after acute resistance exercise [20] - 2-7 days after acute eccentric exercise (knee flexion ranged from 40º to 110º) [21] - 24 h after prolonged exercise (100 km race) [22]
Lactate dehydrogenase [23]	2-fold	- 3-5 days after eccentric exercise (eccentric exercise of the forearm flexors) [24]
Aldolase A [25]	6-fold	- 3-5 days after acute eccentric exercise (one-leg calf-raise exercise) [26]
Aspartate transferase [27]	1.1-fold	- Immediately after exercise until 24 h (21 km run) [28]
Myoglobin [29]	4-fold	- 30 min to 5 h after exercise (a soccer match) [30]
Carbonic anhydrase III [31]	3-fold	- 24 h after 21 km running [32]
Immunoassay-based muscle damage biomarker panel		
Skeletal troponin I [33]	5-fold	- 6-24 h after 20 min downhill running [34]
Myosin light chain 3 [16]	2.15-fold	- 4 and 7 h after inducing localized skeletal muscle damage after anesthesia [16]
Fatty acid–binding protein 3 [16]		- 2 h after inducing localized skeletal muscle damage utilizing anesthesia [16]
Creatine kinase measured by a mass assay [16]	9.7-fold	- 7 h after isoproterenol-induced myocardial injury [16]

2.2.1. Creatine Kinase

Creatine kinase (CK) is a dimeric globular protein consisting of two subunits with a molecular mass of 43-45 kDa for each subunit. CK buffers cellular adenosine triphosphate (ATP) and adenosine diphosphate concentrations by catalyzing the reversible exchange of high-energy phosphate bonds between phosphocreatine and adenosine diphosphate produced during muscular contraction. At least five isoforms of CK exist: three isoenzymes in the cytoplasm [CK-MM (found in skeletal muscle and heart), CK-MB (found in the heart and rises when heart muscle is damaged) and CK-BB (found mostly in brain)] and two isoenzymes (non-sarcomeric and sarcomeric) produced in the mitochondria that are increased in mitochondrial myopathies [35]. The cytoplasmic isoenzymes (CK-MM, CK-MB, CK-BB) provide specific information on injured tissue because of their tissue distribution. CK-MM is found in several domains of the myofiber where ATP consumption is high and is a biomarker of muscle disease [36]. Strenuous exercise that damages skeletal muscle cell structures at the level of sarcolemma and Z-disks [27] results in an increase in total CK [19] in serum. When exercise intensity is within the normal range of metabolism, the muscle tissue is exercised without significant changes in membrane permeability. However, when exercise intensity exceeds this range, permeability changes and CK appears in the circulation [37].

The linkage of these biomarkers or their pattern of appearance to specific damage etiologies is incompletely defined. As a result, the interpretation of an elevated serum CK concentration is challenging in an individual in whom the etiology of muscle damage is unclear. The CK elevation may have resulted from a self-limited process like the transient injury of recent exercise [38], underlying muscle disease [39] or drug-induced muscle damage [40]. Additionally, an elevated CK concentration does not indicate the age of the damage, or if the process is ongoing and thus exposing the individual to a risk of subsequent rhabdomyolysis [41].

2.2.2. Lactate dehydrogenase

Lactate dehydrogenase (LDH) is an enzyme involved in anaerobic glycolysis that interconverts pyruvate and lactate, with concomitant interconversion of nicotinamide adenine dinucleotide and is critical for meeting rapid high-energy demands [42]. There are normally five isoenzymes (LDH1, LDH2, LDH3, LDH4, LDH5) expressed in living

cells, which are made of the combination of M-polypeptide chains and H-polypeptide chains. M chains catalyze the conversion of pyruvate to lactate, while H chains improve the aerobic oxidation of pyruvate [27]. Serum LDH activity is a biomarker of cell damage, and the specific increase in isoenzymes (such as in LDH5 [43]) may be helpful for the diagnosis of non-traumatic acute rhabdomyolysis [27]. Exercise induces a significant increase in LDH and the degree of increase depends on the intensity and duration of exercise [27]. An eccentric bout of exercise induces a much greater increase in serum enzyme activity (showing more muscle damage) than concentric exercise [24].

2.2.3. Aldolase

Aldolase has a molecular weight of 160 kDa and is a glycolytic enzyme which catalyzes the reversible cleavage of fructose 1-6-biphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in the glycolysis metabolic pathway [44]. Aldolase is localized in the cytoplasm and in the cell nucleus, where it is located in the heterochromatin region [44]. In vertebrate tissues, three aldolase isoenzymes have been observed: the muscle isoenzyme, aldolase A, the liver isoenzyme, presents also in the kidney, aldolase B, and the brain isoenzyme, aldolase C [44]. The isoform of fructose-1,6-bisphosphate aldolase that binds to the actin-containing filament of the cytoskeleton is aldolase A (muscle type), exhibits a tissue specific binding pattern [27]. An increase in serum aldolase is found in myotonic muscle disease, such as progressive muscular dystrophy and polymyositis [27]. Isolated serum aldolase A is also recommended as a biomarker of damaged early regenerating muscle cells in myositis patients [45].

2.2.4. Aspartate transaminase/aspartate aminotransferase

Aspartate transaminase (AST) is an aminotransferase that catalyzes the reaction: aspartate + α-ketoglutarate → oxaloacetate + glutamate. This reaction occurs between the mitochondria and cytosol and provides energy to the cells in the form of ATP. The enzyme, localized primarily in the skeletal and myocardial muscle, liver and erythrocytes, has a molecular weight of 90 kDa and is mainly a biomarker of liver disease [27]. AST activities also increase immediately after exercise as well, remaining elevated for 24 h [28]. The rise is also related to the duration of exercise [46], and it can occur even without clinical symptoms. Even moderate-intensity exercise of

prolonged duration can induce asymptomatic exertional rhabdomyolysis [47]. CK and AST activity assays work well for detecting higher grade myopathies but are poor predictors of low-grade histopathology findings [48].

2.2.5. Myoglobin

Myoglobin is an abundant haem-containing oxygen (O_2) carrier expressed predominantly in type I skeletal muscle fibers and is a clinically useful muscle damage biomarker [49]. Following strenuous exercise, myoglobin is released as a result of degradation of protein structures within muscle [50]. Plasma, serum and urine myoglobin measurements might be useful for identifying minor skeletal muscle damage [51]. Myoglobin has been declared as a "gold standard" for prognosis, especially in patients with non-traumatic rhabdomyolysis [52].

2.2.6. Carbonic Anhydrase III

Carbonic anhydrase III (CA III) is a zinc metalloenzyme known to catalyze the reversible hydration of carbon dioxide (CO_2) to bicarbonate ions. CA III is approximately 300-fold less enzymatically active compared with the most efficient carbonic anhydrases. Hence, its physiological relevance is still unclear, however, it has been proposed to be associated with muscle contraction, protection from oxidative stress, and regulation of adipogenesis [53]. CA III is another useful indicator of muscle damage because it is present in skeletal muscle but not in the myocardium, and is released into the circulation following damage [54]. It is clinically applicable as a diagnostic biomarker for muscle disease, and probably reflects type I fiber abnormalities, with greater sensitivity than CK and aldolase [31].

2.3. Immunoassay-based muscle damage biomarker panel

Through the Critical Path Institute's Predictive Safety Testing Consortium, a novel immunoassay-based muscle damage biomarker panel has been evaluated that includes the analytes skeletal troponin I (sTnI), myosin light chain-3 (Myl3), fatty acid-binding protein-3 (FABP3), and CK measured by a mass assay (CKm). These analytes can be measured using serum and/or plasma samples and commercially available reagent systems [16]. Goldstein [16] evaluated the release and clearance dynamics of muscle damage biomarkers using a panel that includes CK, AST activity, and immunoassay-based muscle damage biomarker panel. Acute localized muscle injury

was induced using Marcaine, and blood samples were collected at various intervals. The goal was to compare biomarker kinetics after injury. Results showed similar timing between immunoassay-based muscle damage biomarker panel (sTnI, Myl3, FABP3) and traditional biomarkers (CK, AST activity), with elevated levels at 2 hours post-injury and peak concentrations between 4 and 7 hours. The immunoassay-based muscle damage biomarker panel demonstrated a higher dynamic range, indicating improved sensitivity compared to CK and AST activity biomarkers (Figure 2.2).

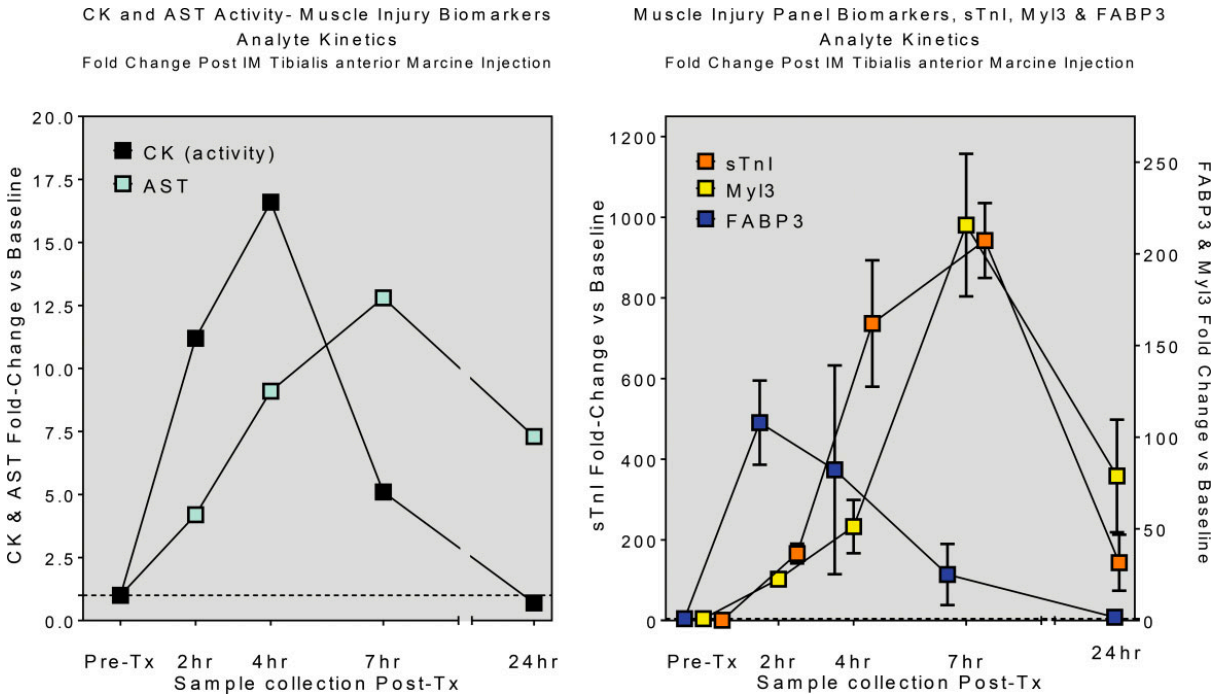

Figure 2.2 Established skeletal muscle injury biomarkers, creatine kinase (CK) and aspartate transaminase (AST) activity (left hand panel), and muscle injury biomarker panel including analytes skeletal troponin I (sTnI), myosin light chain-3 (Myl3), fatty acid-binding protein-3 (FABP3) (right hand panel). These demonstrate similar release and clearance kinetics following acute intramuscular Marcaine induced skeletal muscle injury. The muscle injury biomarker panel demonstrated enhanced dynamic range with greater fold-change increases over baseline values than CK and AST suggesting improved sensitivity to detect skeletal muscle injury [16].

2.3.1. Skeletal troponin I

Troponin I (TnI) is a regulatory protein that plays an essential role in the contraction of skeletal and cardiac muscle. TnI is a troponin complex subunit that inhibits actomyosin-adenosine triphosphatase activity thereby disrupting actin and myosin

interaction during the relaxed state of the muscle [55]. TnI exists in three isoforms: cardiac (cTnI); slow skeletal; and fast skeletal (sTnI). The assay for cTnI is well established, has superior tissue specificity and enhanced diagnostic sensitivity to damage compared with conventional biomarkers of cardiac muscle damage including CK [56, 57]. sTnI is the only skeletal muscle specific protein biomarker included in the muscle damage biomarker panel [16]. sTnI is a component of myofilaments and can exist as 2 isoforms: slow-twitch (Type I) or fast-twitch (Type II) fibers [16]. A reliable skeletal muscle assay for sTnI in blood serum is available [58], which is developed for differential detection of skeletal TnI isoforms in the serum of a patient with rhabdomyolysis [58]. This assay is specific for sTnI only, unlike the previously available experimental immunoenzymatic assay for sTnI in which antibodies cross-react with both sTnI and cTnI [59].

2.3.2. Myosin light chain 3

Myl3 is an essential light chain of the myosin molecule expressed predominantly in cardiac and skeletal muscles. Myosin is a six subunit mechano-chemical enzyme that functions to couple the hydrolysis of ATP to conformational changes that result in movement of the protein complex along actin filaments. This hexameric protein consists of two heavy chain subunits, which form the head and tail domains of the complex. The heavy chains are held together by four light chain subunits. The light chain subunits consist of two regulatory light chains with phosphorylation sites and two essential light chains. Following damage to muscle tissue, the constituent subunits of myosin become dissociated, and Myl3 is released into the blood stream. To date, efforts have focused on determining the utility of Myl3 as a biomarker of cardiomyocyte damage [60]. Given its abundant expression in type I skeletal muscle fibers, it has been noted that Myl3 may also be useful as a circulating surrogate for damage to type I myocytes [60]. Tonomura et al. [61] investigated the change in circulating Myl3 in response to a panel of cardiac and skeletal muscles toxicants. Myl3 accurately detected damage, but could not differentiate between skeletal and cardiac muscle toxicity. In the same study, rats treated with a panel of hepatobiliary toxicants had no measurable increase in plasma Myl3. As Myl3 is found in both cardiac and skeletal muscle fibers, increases in Myl3 should be interpreted in conjunction with other specific skeletal muscle and cardiac biomarkers (e.g., cTnI) [62].

2.3.3. Fatty acid–binding protein 3

FABP3 is a cytosolic lipid transport protein and is abundant in skeletal muscle and cardiac muscle but is also present in brain, liver, and small intestine [16]. The protein encoded by FABP3 has a predicted amino acid length of 133 and a molecular mass of 14.9 kDa, and cytoplasmic subcellular localization. FABP3 plays a permissive role in the transport and mobilization of fatty acids within the cellular environment. FABP3 binds both saturated and polyunsaturated fatty acids with high affinity. It is thought to shuttle fatty acids from the plasma membrane to intracellular sites of usage including the ß-oxidation machinery. FABP3 expression is increased in response to physiological conditions that increase fatty acid demand and availability, such as increased testosterone production, endurance training, and malnutrition [63]. Therefore, FABP3 has been proposed as a biomarker of both cardiac and skeletal muscle damage and has been previously shown to correlate with skeletal muscle degeneration [64].

Although much is known regarding exercise-induced skeletal muscle damage of the peripheral muscles, very little is known about damage to the respiratory muscles. Therefore, the following sections will focus on the respiratory system, muscles, and respiratory muscle damage.

2.4. The respiratory system

The human respiratory system is shown in Figure 2.3 and is a complex arrangement of anatomical structures and physiological mechanisms that coordinate the exchange of O_2 and CO_2, which are essential for the maintenance of life [65]. Starting from the upper respiratory tract, the nasal cavity plays a crucial role in the process of conditioning inspired air. The mucous membrane, which has a high concentration of ciliated and goblet cells, creates an environment that promotes the processes of humidification, filtration, and warming of entering air [65]. The nasal conchae and meatuses exhibit architectural subtleties that result in the induction of turbulent airflow, thereby optimizing the interface between the mucosa and the air for the purpose of efficient air cooling. Moreover, the nasal cavity is equipped with a robust defense mechanism against pathogens due to the presence of immunoglobulins and lysozymes in the released mucus [65].

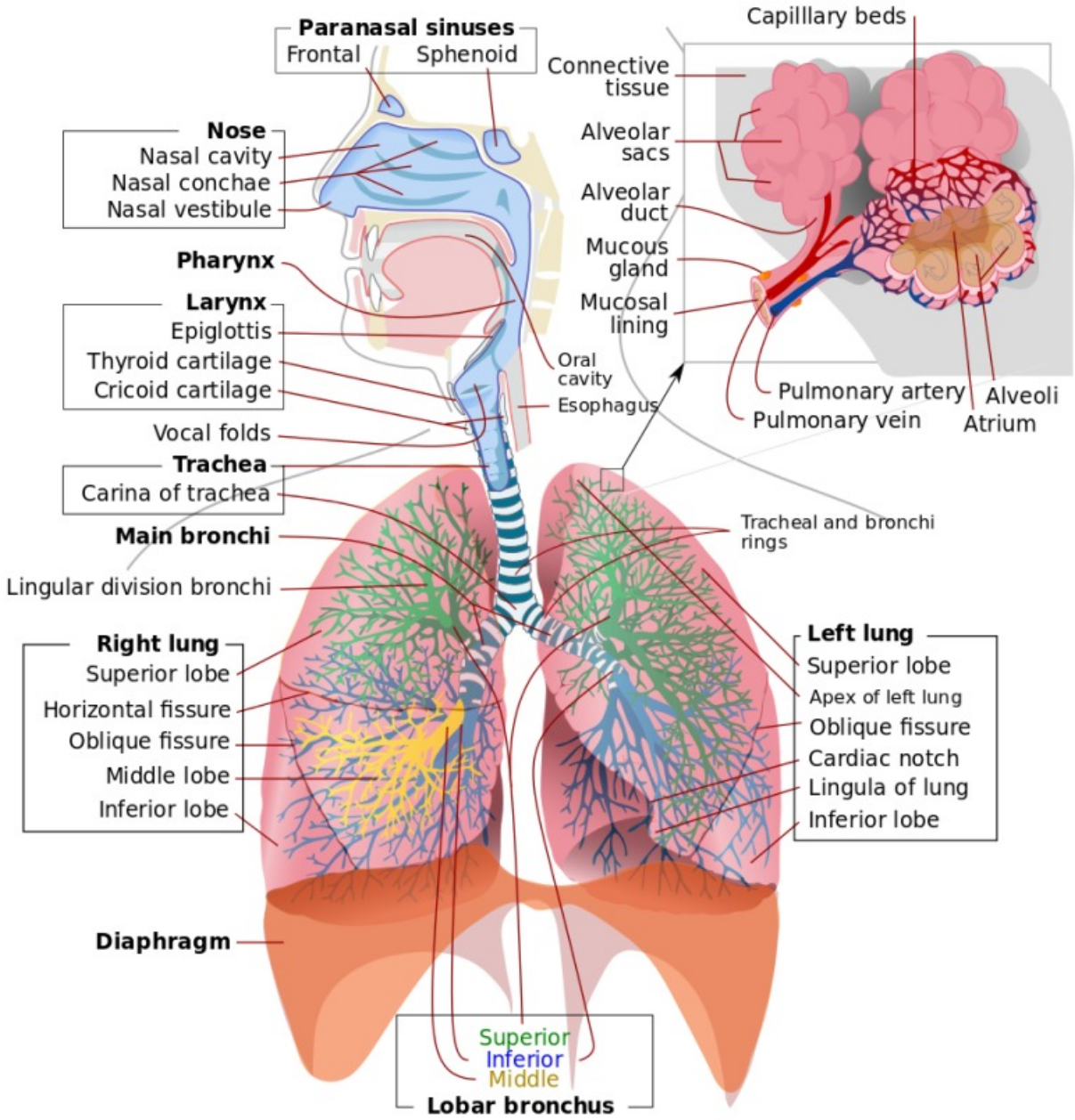

Figure 2.3 The respiratory system consists of the airways, the lungs, and the respiratory muscles that mediate the movement of air into and out of the body [66].

Progressing through the upper respiratory tract, the pharynx assumes a pivotal role as a crucial intersection for both the respiratory and digestive systems. The conduit, which consists of the nasopharynx, oropharynx, and laryngopharynx, facilitates a smooth passage of air to the lower respiratory tract while also serving as a barrier between the respiratory and digestive systems [65]. The larynx, located in a lower position, contains the vocal cords and functions as a protective mechanism against

the inhalation of food into the lower respiratory pathways [65]. The trachea, which is composed of fibrocartilage, facilitates the movement of air from the larynx to the bronchi as it progresses along the lower respiratory tract. The tracheal rings, composed of hyaline cartilage, provide structural stability, thereby limiting collapse in response to changes in intra thoracic pressure [65]. The primary function of the epithelial lining, which is mainly composed of ciliated cells, is to aid the coordinated transport of mucus and particles towards the upper airways, hence enhancing the respiratory defense systems [67].

The trachea can be further divided into the bronchial tree, which consists of a complex network of airways that penetrate the pulmonary parenchyma. The primary bronchi, which subsequently divide into secondary and tertiary bronchi, constitute this complex anatomical network [67]. The terminal bronchioles, which lack cartilaginous support, terminate in clusters of alveoli, which are the essential structures responsible for gas exchange in the lungs [67]. Gas exchange primarily occurs within the alveoli, which are surrounded by a vast network of capillaries. The intricate process described above is made possible by a respiratory membrane that is characterized by a varying thickness [67]. This unique feature allows for the efficient exchange of O_2 from the alveoli to the circulation, while simultaneously enabling the removal of CO_2 in the opposite direction [67].

The pleura, a bilayer membrane envelope that surrounds the lungs, plays a crucial role in facilitating the smooth movement of respiratory structures during the process of ventilation [68]. The visceral pleura demonstrates a close adherence to the surface of the lung, whereas the parietal pleura serves to line the inner surface of the thoracic cavity. The presence of a serous fluid layer between the pleural layers serves to reduce friction, hence promoting smooth movement of the lungs during breathing cycles [65].

The pulmonary vasculature is responsible for regulating the blood supply to the lungs. The deoxygenated blood originating from the right ventricle is directed towards the pulmonary arteries, which then passes via the pulmonary capillaries surrounding the alveoli where the process of oxygenation occurs. Subsequently, oxygenated blood is sent back to the left atrium via the pulmonary veins, ready for systemic circulation [69].

The regulation of breathing is controlled by the medullary respiratory centers located in the medulla oblongata. These centers effectively regulate the rhythm and pattern of respiration, adaptively responding to changes in blood gas concentrations, namely CO_2 levels [66]. In addition, the peripheral chemoreceptors, which are located in the carotid bodies and aortic arch, play a role in the precise control of respiratory dynamics by monitoring the amounts of O_2 in the blood [70].

While humoral mechanisms play a vital role in maintaining respiratory homeostasis at rest, the respiratory system's response to exertion involves more complex interactions [71], including central and peripheral neurogenic pathways [71]. Central neurogenic mechanisms involve the integration of sensory input from receptors located in various parts of the body, including the lungs, muscles, and joints, and by the respiratory centers in the brainstem [72]. Peripheral neurogenic mechanisms encompass the reflexive responses initiated by receptors located in the airways, lungs, and chest wall [72]. Understanding the interplay between humoral, central neurogenic, and peripheral neurogenic pathways is essential for comprehensively elucidating respiratory control mechanisms, particularly during dynamic conditions such as exertion [71].

2.5. The respiratory muscles

The respiratory muscles are shown in Figure 2.4 and play a crucial role in facilitating the fundamental physiological function of the lungs, which is to facilitate gas exchange by delivering O_2 to the bloodstream and eliminating CO_2 from it. The muscles responsible for the process of ventilation, which involves the passage of air into the lungs, are referred to as pump muscles. In contrast, the airway muscles are responsible for regulating the diameter of both the upper and lower airways. These muscles consist of both skeletal muscles, which are found in the upper airways, and smooth muscles, which are present in the trachea and bronchi. The respiratory muscles are required to undergo structural adaptations in response to diverse environmental and pathological circumstances. Similar to other skeletal muscles, these muscles possess structural plasticity (i.e., changes in their size, composition, and function in response to various stimuli), enabling them to alter their functionality accordingly [73]. For example, during conditions that demand increased respiratory effort, such as exercise or lung disease, the respiratory muscles may undergo hypertrophy, increasing in size and strength to meet the elevated demand for

ventilation [74]. Conversely, in situations of prolonged inactivity or respiratory muscle disuse, such as prolonged bed rest or mechanical ventilation, the respiratory muscles may undergo atrophy, leading to decreased muscle mass and function [75].

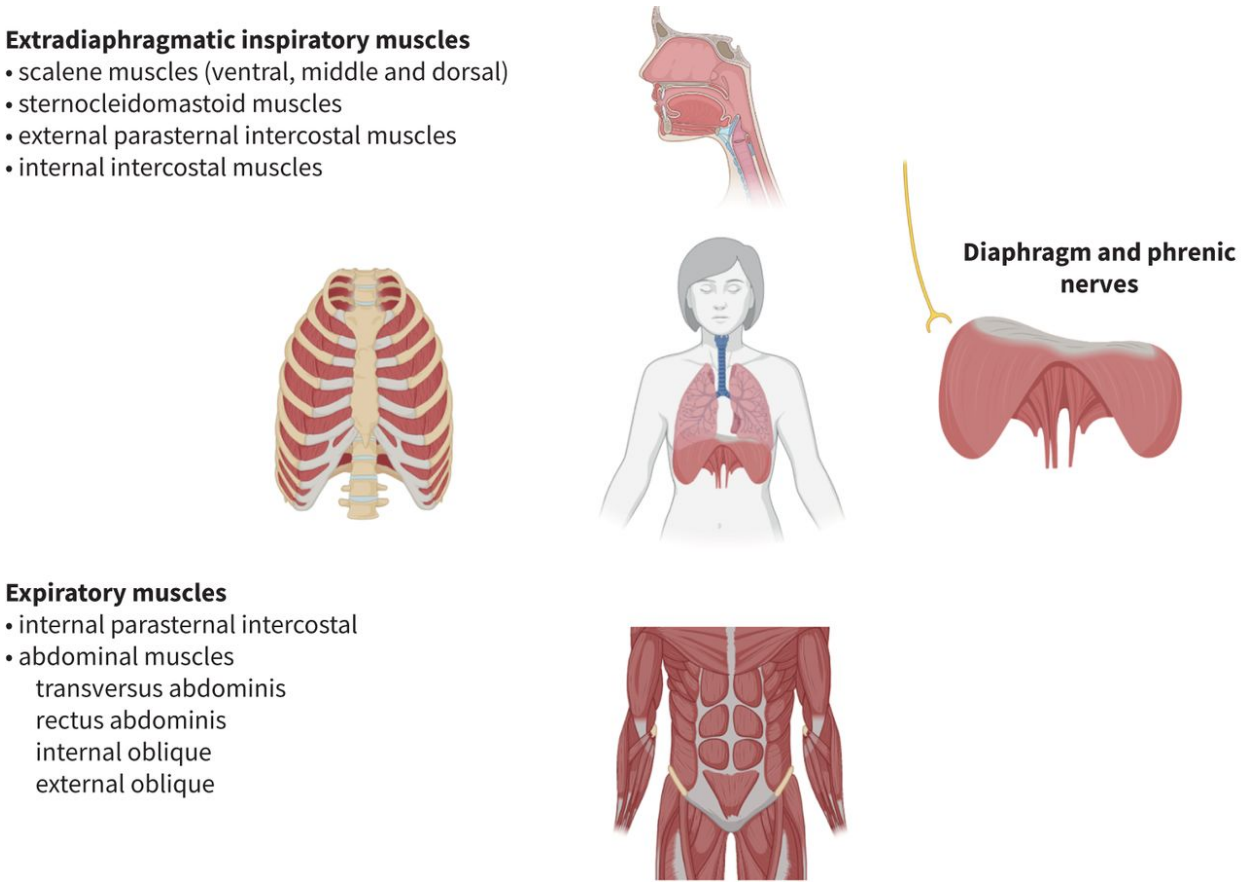

Figure 2.4 The respiratory muscles involved in the generation of respiration [76].

The respiratory muscles are a type of skeletal muscle consisting of the inspiratory and expiratory muscles and are responsible for ventilation (Figure 2.5) [76]. The muscles that are constantly engaged during inspiratory or expiratory actions are classified as "primary" respiratory muscles [77]. The muscles that are occasionally engaged during heightened inspiratory or expiratory efforts are referred to as accessory respiratory muscles. The categorization of primary and accessory respiratory muscles may exhibit variation among different species. The main muscles responsible for inspiration in humans are the diaphragm, external intercostals [78] and parasternal intercostal muscles [77], which work together to expand the chest wall. Accessory muscles, including the sternocleidomastoid, scalenes, and triangularis sterni, are involved in the movement of the chest wall. These muscles are considered accessory since they are

only activated during heightened inspiratory exertion. Indeed, the activation of these supplementary inspiratory muscles is a significant clinical indicator of inspiratory loading [68]. In the context of human physiology, expiration is commonly seen as a passive process that does not necessitate muscle engagement. Instead, it is primarily facilitated by the elastic recoil of both the lung and chest wall. During the process of forced expiration, the activation of abdominal muscles is observed, leading to an increase in intraabdominal pressure [65]. Consequently, abdominal muscles are categorized as accessory respiratory muscles, and their activation is frequently employed in clinical practice as a measure of respiratory load [65].

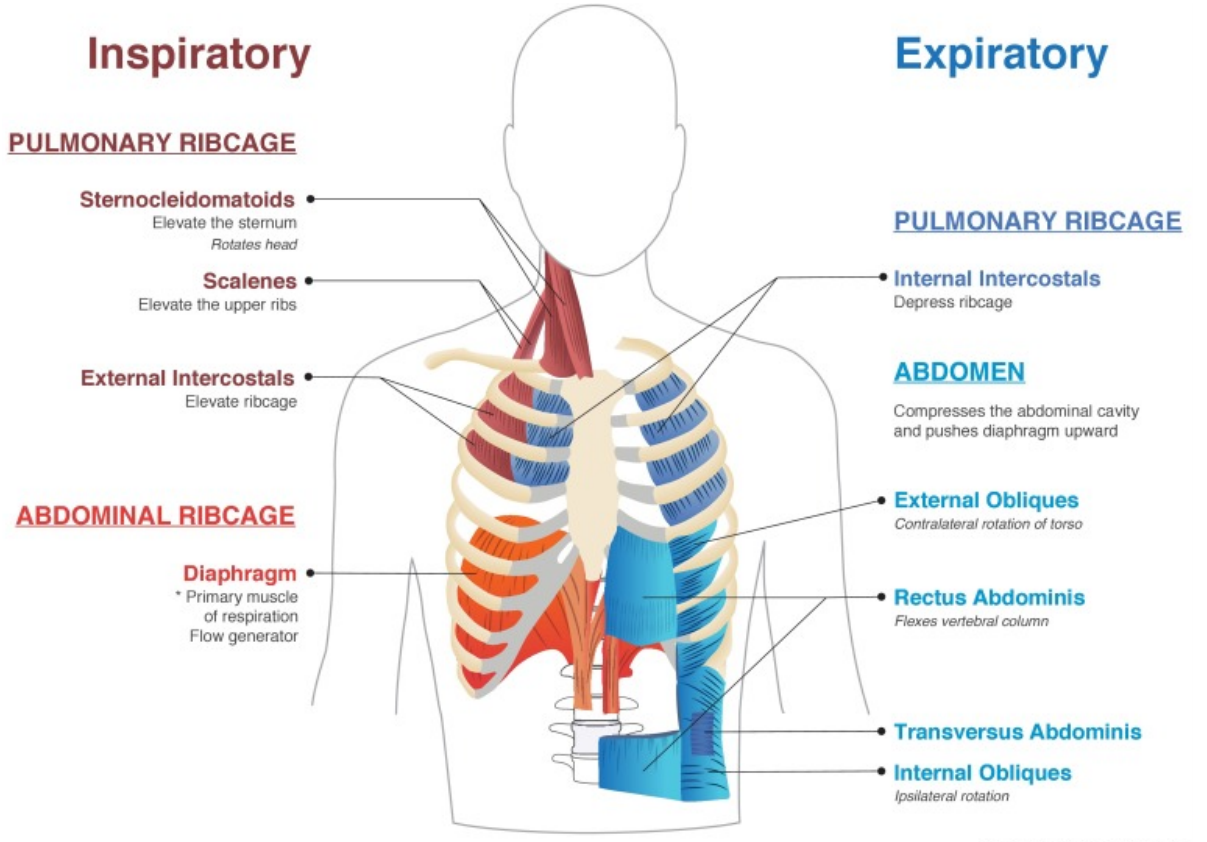

Figure 2.5 Muscles of inspiration and expiration [79].

The primary muscle of respiration is the diaphragm and it is responsible for about 70-80% of respiratory work during quiet breathing. The diaphragm muscle is a narrow, dome-shaped muscular structure that is attached to the lower ribs and the lumbar vertebrae (i.e., the crural diaphragm) and functions as a partition, dividing the thoracic (pleural) and abdominal (peritoneal) cavities within the human body [73]. The diaphragm is classified into three distinct sections based on the origin of its muscular

fibers [80]. The muscle fibers located in the sternal area have their point of origin at the xiphisterna junction. The fibers present in the costal region have their origin in the lower rib cage [80]. The fibers located in the crural area have their origin in the upper lumbar vertebrae. The fibers in each of these sections of the diaphragm muscle are inserted into the central tendon. In the sternal area, the arrangement of fibers typically exhibits a parallel orientation. In the coastal region, it is observed that the circumference of the costal edge exceeds that of the central tendon insertion. Consequently, the fibers in this area have a radial orientation that extends outward from the central tendon. The arrangement of fibers in the crural region is somewhat intricate due to their involvement in encircling the esophagus and functioning as an esophageal sphincter [80]. The mechanical consequences of fiber activation in distinct regions of the diaphragm muscle are contingent upon the precise origins and insertions of the fibers, as well as the diverse stresses exerted by ribcage and abdominal displacement [81].

The respiratory muscles display two unique features compared to peripheral skeletal muscles. Firstly, the work of the respiratory muscles is low in intensity but constant in duration. Therefore, there is no opportunity for complete rest [2]. This is in comparison to peripheral skeletal muscles, which experience a mixture of fairly high intensity loading for the duration of a few minutes to hours daily, followed by a period of rest with no loading or much lighter loads. Secondly, the morphological configuration of the diaphragm differs from peripheral skeletal muscles. The diaphragm is a heterogeneous muscle and the costal and crural parts show two clearly defined, anatomically separate segments. They have a distinct neural innervation, neuro-motor control and mechanical action, which allows the costal and crural parts to vary in contraction in each region and sub-regions [82, 83]. During apparent "concentric" contractions, the geometry of the diaphragm may tolerate regional lengthening, which may increase its vulnerability to eccentric damage by relatively light loads [84].

2.6. Respiratory muscle damage

The potential causes of respiratory muscle damage are shown in Figure 2.6. Respiratory muscle damage may occur during and following excessive loading which exceeds the usual requirements of the muscle. Excessive loading can be categorized in two ways - overload or overactivity. Overload is a condition when the force

requirement is higher than usual [85] and this can occur during growth or exercise. Overactivity, in contrast, can be defined as increased work when the firing of the motor neuron is increased beyond its normal physiological levels or duty cycles [85]. Overactivity occurs during many endurance activities and in several disease conditions such as spasticity. In respiratory disease, the respiratory muscles can experience both overload and overactivity because each breath may require a higher inspiratory muscle force and a higher breathing frequency (i.e., number of respiratory muscle contractions per minute). Excessive loading may occur if overload or overactivity is imposed, or if weakness causes the respiratory muscles to require a greater proportion of their maximal strength. Excessive loading of the respiratory muscles can result from several conditions.

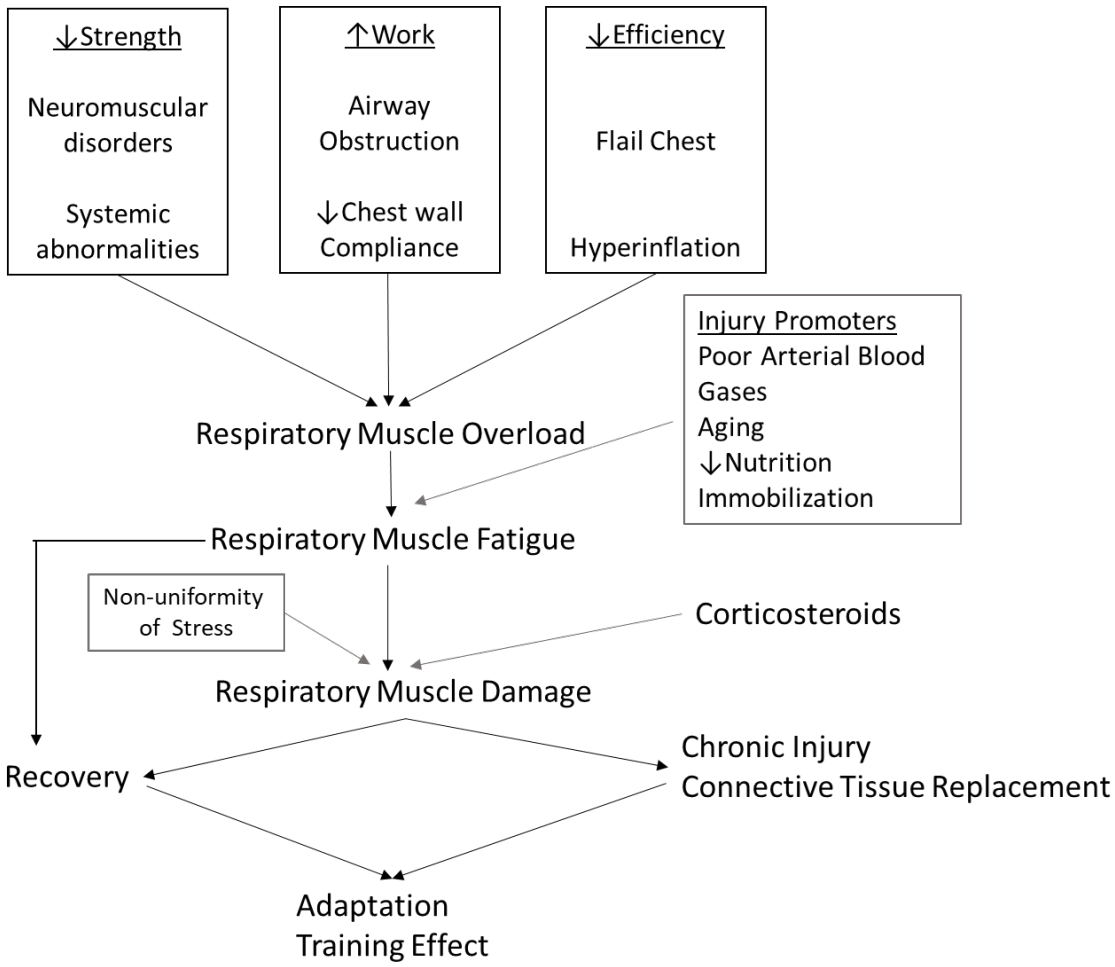

Figure 2.6 Causes of respiratory muscle damage [86].

2.6.1. Respiratory muscle damage and dysfunction in respiratory diseases

In some respiratory diseases including chronic obstructive pulmonary disease (COPD) and asthma, the respiratory muscles may undergo damage [87]. COPD is a complex respiratory condition marked by an ongoing and gradual obstruction of airflow, primarily during expiration [88]. The pathophysiology of COPD is characterized by persistent inflammation of the airways and lung tissue, primarily caused by exposure to harmful particles including cigarette smoke [88]. The persistent inflammation generates a cascade of events that contribute to structural alterations in the airways and lung tissue [89]. These encompass excessive production of mucus, structural changes in the airways, and damage to the walls of the alveoli. As a result, the lungs have less elastic recoil and there is an increase in the resistance of the airways [89]. These modifications have a substantial effect on the process of breathing and impose a greater burden on the respiratory muscles [88, 89].

The diaphragm, being the primary muscle responsible for inspiration, is especially impacted. The increased expiratory flow limitation results in a greater work of breathing [90, 91]. This can result in an increase in the size of the diaphragm and hyperinflation. However, with prolonged duration, the diaphragm may experience fatigue and dysfunction [90]. Diaphragm dysfunction in COPD is characterized by structural and functional alterations. These changes encompass both negative and positive modifications in the diaphragm's structure, influencing its function. The diaphragm's performance is intricately linked to its physiological characteristics at the structural level in COPD patients [92] (Figure 2.7). As COPD advances, additional muscles including the intercostals are used to assist with breathing, increasing the overall muscular burden [89, 91].

The repercussions of respiratory muscle damage and dysfunction in COPD are significant. Dyspnea is a common occurrence in individuals, particularly when they engage in physical activity, as the respiratory muscles face difficulty in meeting the increased need for breathing [91]. Chronic muscle fatigue restricts the ability to engage in physical activity and can lead to a sedentary lifestyle, and worsening physical decline [93]. Furthermore, the heightened energy consumption linked to difficult breathing can lead to systemic consequences, such as weight loss and muscular atrophy [91, 93].

In COPD, the increased expiratory load due to narrowed airways leads to air trapping in the lungs [94]. This prolonged air trapping causes hyperinflation, which shifts the position of the diaphragm downwards and flattens it over time [94]. Additionally, chronic inflammation and structural changes in the lung tissues further weaken the diaphragm's function [95]. Consequently, the diaphragm's ability to contract effectively during inspiration is compromised, contributing to respiratory muscle dysfunction in COPD [94, 95].

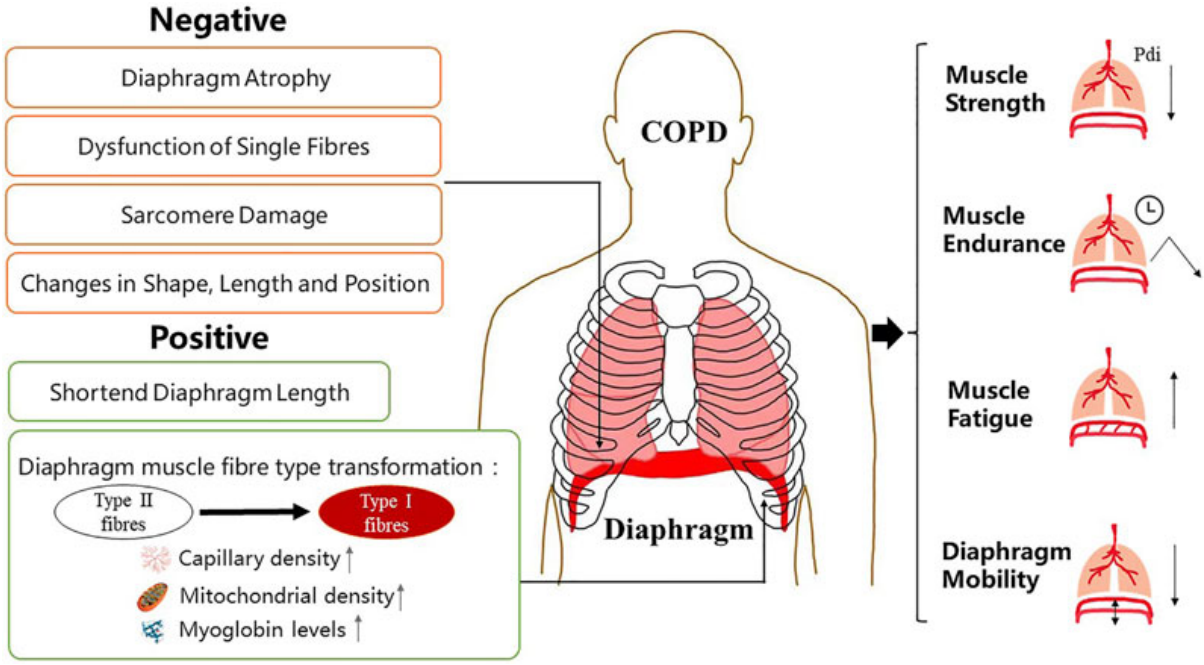

Figure 2.7 Manifestations of diaphragm dysfunction in chronic obstructive pulmonary disease (COPD). Diaphragm dysfunction in patients with COPD is mainly manifested in structural and functional changes. Changes in diaphragm structure include both negative and positive changes. The function of diaphragm depends largely on its physiological characteristics at the structural level [92].

Asthma, a chronic inflammatory condition of the airways, is a distinctive pathophysiology characterized by reversible airflow obstruction and bronchial hyperresponsiveness [88, 96]. Central to asthma is the persistent inflammation of the airways, characterized by various immune cells and mediated by inflammatory molecules [88]. These inflammatory events trigger bronchoconstriction, increased mucus production, and airway edema, collectively contributing to the hallmark symptoms of wheezing, coughing, and dyspnea. In asthma, the dynamic nature of

airway obstruction sets it apart from COPD [96]. Bronchoconstriction, resulting from the contraction of smooth muscles surrounding the airways, is a reversible process, distinguishing asthma exacerbations from the more irreversible airflow limitation seen in COPD [88]. However, the chronic and recurrent nature of asthma exacerbations can lead to long-term structural changes in the airways, known as airway remodeling [96, 97].

The respiratory muscles are intimately involved in the pathophysiology of asthma [97, 98]. During an asthma exacerbation, the increased resistance within the airways necessitates increased respiratory muscle work to maintain sufficient airflow [98]. The diaphragm, being the principal muscle of inspiration, is particularly engaged in overcoming the increased resistance during inspiration [97]. Additionally, accessory muscles such as the intercostal muscles may be recruited to further support the respiratory effort [99]. The increased work of breathing in asthma can result in respiratory muscle damage over time. Individuals experiencing frequent exacerbations may find themselves breathless and fatigued, especially during physical activity [98, 99]. However, unlike COPD, where the irreversible damage to the airways is a predominant feature, the potential for reversibility in asthma allows for better management and control of symptoms with appropriate treatment and medications [97, 100].

From the chronic inflammation and irreversible airflow limitation in COPD to the reversible bronchoconstriction of asthma [98], the strain on respiratory muscles is a common thread. This respiratory muscle damage may contribute substantially to symptoms, exercise limitation, and diminished quality of life, emphasizing the critical impact of muscular involvement across diverse respiratory pathologies [98, 101].

2.6.2. Respiratory muscle damage in COVID-19

Coronavirus disease (COVID-19) is an infectious disease caused by the SARS-CoV-2 virus. The novel virus was first identified in an outbreak in the Chinese city of Wuhan in December 2019 and subsequently identified in Australia in 2020 during the first year of this PhD. COVID-19 can reduce respiratory muscle function and functional capacity [102]. This can be through the direct action of the virus (severe acute respiratory syndrome coronavirus 2) in the respiratory muscles causing muscle cell damage,

inflammation and impaired muscle function (Figure 2.8). This can also be by the immune system responding to the virus leading to acute hypoxic respiratory failure, pneumonia and/or acute respiratory distress syndrome [103, 104].

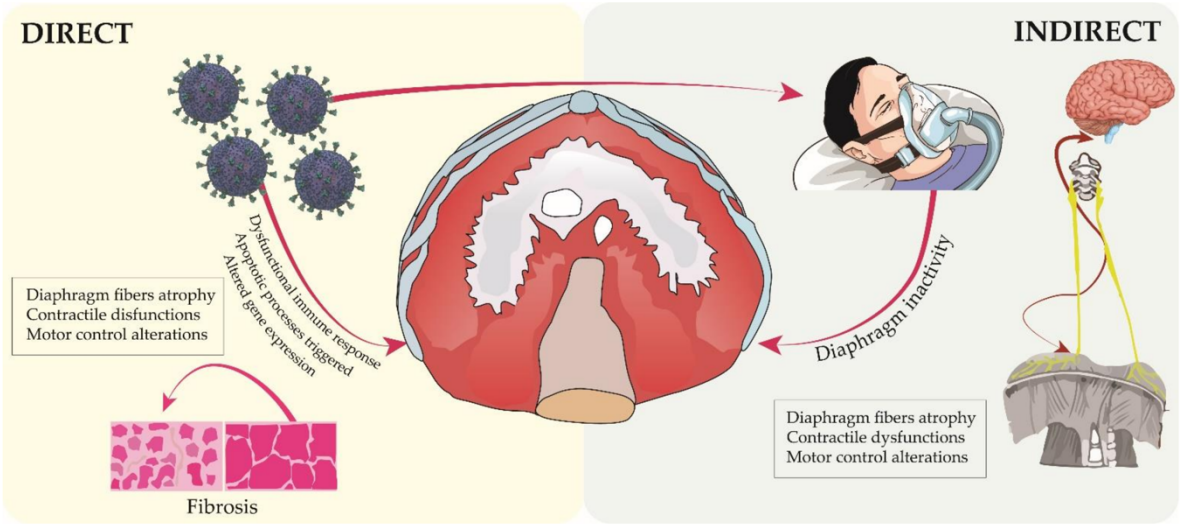

Figure 2.8 Graphical representation of the direct and indirect influence of SARS-CoV-2 infection on diaphragm structure and function. Viral infiltrations and infection (direct mechanism) may trigger a dysfunctional immune response and myokines release from diaphragm and adjacent muscles leading to a rise in localized inflammation. The viral infection may further induce alterations in gene expression, with upregulation of genes involved in tissue fibrosis. Further, apoptotic mechanisms are upregulated, culminating in an altered diaphragmatic structure. Additionally, severe COVID-19 may lead to mechanical ventilation (indirect mechanism) triggering all the dysfunctional processes associated with muscle inactivity. This leads to further alterations to diaphragm structure and function [105].

The respiratory muscle damage in COVID-19 is a result of a complex interplay of several pathological mechanisms [106]. One significant factor contributing to this damage is the direct invasion of respiratory epithelial cells by the SARS-CoV-2 virus [106]. The virus utilizes the angiotensin-converting enzyme 2 receptor, which is abundantly present in the respiratory tract, as an entry point [107]. This direct viral invasion triggers a cascade of inflammatory responses in the affected tissues [106, 108]. In severe cases, the inflammation can extend to the respiratory muscles, causing cellular damage and impairing their normal function [107]. The exaggerated immune response seen in severe COVID-19 cases, often referred to as a cytokine storm, plays

a pivotal role in respiratory muscle damage. The release of an excessive amount of pro-inflammatory cytokines, including interleukin-6 and tumor necrosis factor-alpha, can lead to systemic inflammation [107, 108]. The respiratory muscles, being an integral part of the respiratory system, are vulnerable to this inflammatory cascade [106, 107]. Prolonged exposure to high levels of cytokines can result in tissue damage and compromise the contractile function of the respiratory muscles [107, 108].

Another contributing factor is the potential for microvascular thrombosis in COVID-19 [109, 110]. The virus has been associated with a higher risk of blood clot formation, and these clots can affect blood vessels supplying the respiratory muscles. Microvascular thrombosis leads to reduced blood flow, causing ischemia and depriving the muscles of O_2 and nutrients [110]. The resulting lack of perfusion can contribute to muscle injury and impair their ability to generate force effectively. The systemic nature of COVID-19 further complicates the scenario [106, 110]. The prolonged illness and the body's efforts to combat the virus can lead to muscle wasting and weakness, including the respiratory muscles [106]. This generalized impact on muscle tissue can extend the recovery period and increase the risk of persistent respiratory symptoms even after the acute phase of the infection has resolved [109]. In summary, COVID-19-induced respiratory muscle damage arises from a combination of direct viral invasion, cytokine-driven inflammation, microvascular thrombosis, and systemic effects leading to muscle wasting [106, 109, 110].

The implications of respiratory muscle damage in COVID-19 are significant [105]. Severe cases may progress to respiratory failure, necessitating mechanical ventilation to assist with breathing [105, 111]. Even after surviving the acute phase of the illness, individuals may face prolonged recovery characterized by persistent respiratory symptoms and reduced lung function [111]. Rehabilitation and respiratory therapy become crucial components of post-COVID-19 care to help patients regain optimal respiratory function and mitigate long-term consequences [106, 111]. Additionally, the potential for the development of chronic respiratory conditions underscores the importance of comprehensive follow-up care and ongoing monitoring for individuals who have experienced respiratory muscle damage due to COVID-19 [111]. Rehabilitation strategies targeting respiratory muscles in COVID-19 patients involve leveraging the well-established benefits of physical activity and exercise on the

cardiorespiratory system. Physiotherapy and inspiratory muscle training (IMT), previously applied in conditions like COPD, have been adapted for COVID-19 [112]. Tailored rehabilitation plans consider factors such as disease severity, comorbidities, and patient age. Studies indicate positive outcomes, such as improved pulmonary function with IMT and physiotherapy [105, 112]. Comprehensive approaches encompass specific respiratory muscle exercises, full-body physical activity, physiotherapy, and nutritional support [105] (Figure 2.9).

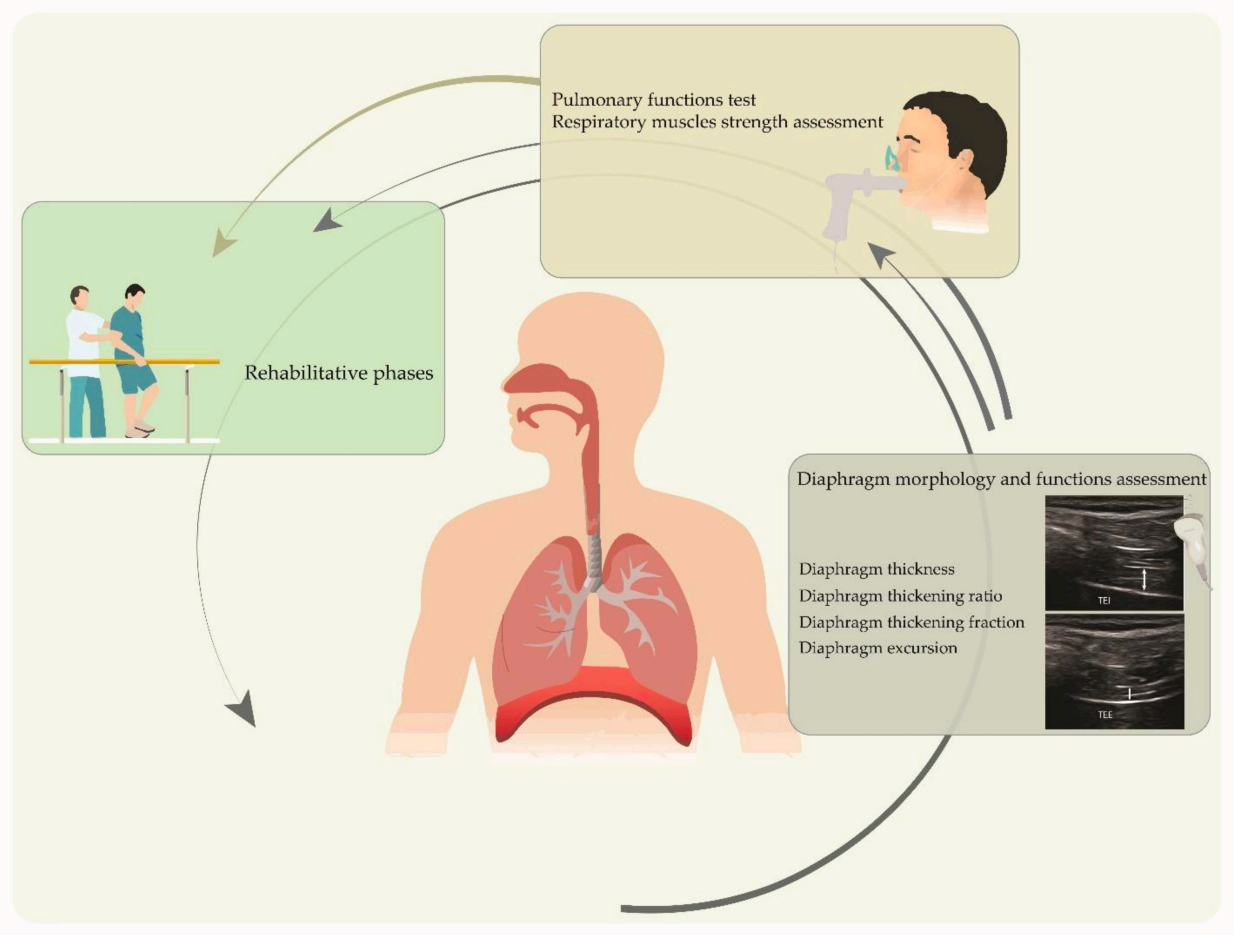

Figure 2.9 Graphical summary of the possible strategies to support and monitoring of COVID-patients healing process [105].

2.6.3. Problems associated with respiratory muscle damage

Because of the essential role in ventilation, respiratory muscle damage may have serious consequences as it leads to respiratory muscle dysfunction [2]. These may range from exercise intolerance and dyspnea through to ventilatory failure where the respiratory muscles can no longer provide enough ventilation to maintain arterial blood

gases within a physiological range [113]. Exercise intolerance and dyspnea can prevent individuals from working and performing many daily activities. Respiratory failure can lead to prolonged hospitalization, intensive care, and eventually death. Respiratory muscle dysfunction has been implicated as a cause of respiratory failure, although its specific etiology is not yet known. It is hypothesized that weakness can lead to respiratory failure [114]. Reid et al. [113, 115] have shown that increased resistive loading in animals over several days is associated with both damage to the diaphragm and hypercapnic respiratory failure. Reid et al. [115] investigated whether ventilatory failure was linked to muscle fiber damage and alterations in myofibrillar proteins. Hamsters underwent ventilatory failure induced by tracheal banding for six days, resulting in respiratory acidosis, hypoxemia, and increased pulmonary resistance. Diaphragm analysis revealed a higher fraction of abnormal muscle and inflammatory cells in banded hamsters. Electron micrographs showed sarcomeric disruption and Z band streaming. While myofibrillar changes were quantitative, not qualitative, sulfhydryl group reactivities were reduced. Calpain digestion of purified myofibrils indicated faster degradation rates for specific proteins. Ventilatory failure induced by resistive loading was associated with diaphragm injury, including changes in myofibrillar complexes and increased susceptibility to calpain-mediated degradation [115].

Another experimental study [116] was conducted to assess respiratory muscle injury in rats exposed to inspiratory resistive loading and to measure the release of sTnI into the blood. This release was correlated with changes in arterial blood gases, respiratory drive, and pressure generation over time. Approximately 1.5 hours into loading, hypercapnic ventilatory failure occurred, followed by diaphragmatic fatigue around 1.9 hours. Loading was stopped at respiratory pump failure, about 2.4 hours after onset. During post-loading occlusions, pressure profiles resembled pre-loading occlusions, indicating potential muscle injury. Western blot analysis revealed the release of fast sTnI during loading, suggesting load-induced injury to fast glycolytic fibers, likely in the diaphragm. This study suggested that the released fast sTnI may offer insights into respiratory muscle dysfunction and serve as a marker for impending muscle fatigue [116]. It is possible that fatigue, weakness, or injury, alone or in concert may contribute to respiratory failure in some cases [86]. Due to these associated clinical

complications, it becomes evident that measuring the level of respiratory muscle damage is important.

2.6.4. Experimental techniques to increase respiratory muscle work

Respiratory muscle work increases during exercise and respiratory disease. During exercise, the increased ventilatory demands increase neural drive to the respiratory muscles, which in turn increases the mechanical work developed by the muscles [117]. Higher intensities of exercise up to maximum increase the mechanical and metabolic stress of the respiratory muscles [117], which may increase the susceptibility to damage [118]. Damage may be worse in patients suffering from respiratory diseases such as COPD [118], because these individuals often exhibit respiratory muscle weakness and reduced respiratory muscle endurance [91]. In COPD patients, respiratory muscle work is increased both at rest and during exercise because of airflow limitation and geometrical changes of the thorax derived from pulmonary hyperinflation which ultimately would lead to respiratory muscle damage [91].

Measuring biomarkers of respiratory muscle damage in the blood following exercise and in patients with COPD is difficult because the exact site of release cannot be assured. For example, in exercise, these biomarkers are also released from peripheral muscles and potentially respiratory muscles. Similarly, in COPD patients, skeletal muscle dysfunction occurs and affects both respiratory and peripheral muscle groups [119], which makes it challenging to measure blood biomarkers specific to respiratory muscles.

To overcome these challenges, respiratory muscle work can be elevated in healthy individuals whilst at rest. The experimental techniques to load the respiratory muscles include various methods to induce increased resistance or restriction during breathing. Resistive loads to breathing involve augmenting airflow resistance, requiring the respiratory muscles to exert more force to inspire air [120]. Pure elastic loads impose purely elastic resistance on the respiratory system, mimicking conditions such as fibrosis, where lung compliance is reduced [121]. Flow-limitation via Starling resistors involves devices that restrict airflow at high flow rates, leading to flow limitation and necessitating greater effort from the respiratory muscles to maintain adequate ventilation [122]. Chest wall strapping physically constrains chest wall expansion,

compelling the respiratory muscles to work harder against this restriction. Each technique artificially loads the respiratory muscles, thereby mimicking conditions like COPD or neuromuscular disorders.

The literature suggests better efficacy and precision in enhancing respiratory muscle work at rest through inspiratory pressure threshold loading (ITL) [123] or volitional hyperpnea (VH) [124] due to their specific mechanisms of action. ITL offers more targeted approach via targeting inspiratory muscles specifically, while VH challenges both inspiratory and expiratory muscles [125]. These techniques isolate the respiratory muscles in such a way, that any changes in systemic blood biomarkers can be assumed to originate from the respiratory muscles.

ITL requires participants to breathe through a device which increases the load of the respiratory muscles (Figure 2.10). Participants are seated with their feet and back supported and elbows resting on a table. The ITL device can be placed on the table and its height adjusted so the participant can reach the mouthpiece with their head and neck in a neutral alignment. The participants wear a nose clip and breathe through a mouthpiece. Adjustable weights are then attached to a plunger with an inspiratory threshold load that has to be met in order for the participant to inspire [33]. ITL is a tightly controlled experimental approach that allows both primary and accessory respiratory muscles to be exercised without peripheral muscle involvement. This technique elevates inspiratory muscle work in individuals while at rest and thus isolates the respiratory muscles in such a way that any changes in systemic blood biomarkers can be assumed to originate from the respiratory muscles.

Another experimental approach that also allows respiratory muscles to be exercised without peripheral muscle involvement is VH performed at rest. VH involves mimicking the breathing and respiratory muscle recruitment patterns achieved during exercise, but whilst the participant is not moving or exercising. Participants are instructed to increase tidal volume and breathing frequency to mimic the minute ventilation achieved during high intensity exercise. An audio metronome paces breathing frequency and real-time visual feedback of tidal volume is provided throughout the test. Isocapnia is maintained by adding CO_2 into the inspiratory circuit in order to maintain resting arterial CO_2 partial pressures [126, 127]. This experimental technique

results in a breathing and respiratory muscle recruitment pattern that is more ecologically relevant to exercise hyperpnea. There are a few limitations and challenges in replicating accurate mechanical characteristics of maximal exercise hyperpnea during VH. While efforts to mimic maximal exercise responses in terms of tidal volume, breathing frequency and duty cycle are still achievable, they often result in mechanically inefficient and non-physiologic outcomes. Voluntary attempts to achieve peak expiratory flow rates can lead to increased expiratory pressures and excessive mechanical work of breathing, demanding a substantial reduction in end-expiratory lung volume below resting levels, further complicating the replication of physiological responses [128, 129].

Trying to replicate reflex regulation of ventilatory output during VH poses significant challenges, particularly in matching the work of breathing. Despite these challenges, precise feedback, such as customized software providing dual visual feedback of flow and pressures, would facilitate a more consistent replication of the exercise work of breathing during mimicking trials. This approach addresses the concerns raised regarding the mechanical differences that may occur in reflex-driven hyperpnea's. Furthermore, literature has reported that while accurately replicating end-expiratory lung volume remains challenging, effective work of breathing matching is still achievable, ensuring reliable estimations of O_2 consumption during VH [128, 129].

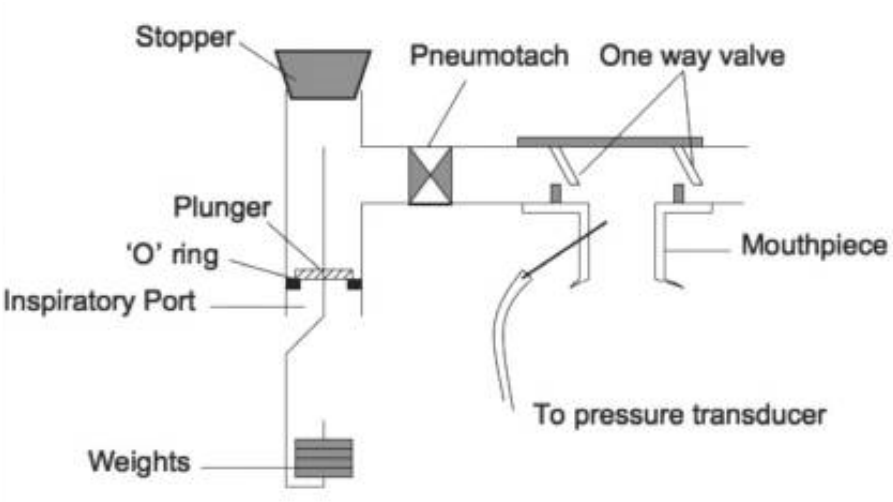

Figure 2.10 Schematic of inspiratory pressure threshold loading device [130].

2.6.5. Measurement of respiratory muscle damage

To date, there is very little information on the measurement of respiratory muscle damage compared to peripheral skeletal muscle damage [27, 131]. A primary reason for this is the difficulty in using many of the methods that are commonly used for peripheral skeletal muscle damage. For example, the use of a muscle biopsy with histopathology has been limited to small samples acquired from patients undergoing thoracic or abdominal surgery [118, 132]. While this method provides direct evidence of structural damage to the muscle, biopsies are invasive and difficult to perform on the majority of respiratory muscles due to their internal location. Furthermore, the small sized biopsies usually miss the damage if it is typically light and patchy. This also limits the opportunity to measure respiratory muscle damage in patients of chronic respiratory diseases and in healthy persons suffering from acute muscle damage occurring due to high ventilatory stress [33].

An alternative, non-invasive approach to detecting muscle damage is the use of MRI (Figure 2.11). Transverse relaxation time (T2) measurements can provide direct information regarding eccentric exercise-induced muscle damage due to its post-exercise elevation [133]. However, MRI of inspiratory muscle damage would be challenging due to the movement of the diaphragm during breathing at rest, the inaccessibility to MRI facilities, and the clarity of image changes post-injury [134].

Respiratory muscle ultrasound is also a safe, repeatable, accurate, and non-invasive bedside technique used to evaluate the anatomy and function of the respiratory muscles [135, 136] (Figure 2.11). Ultrasound finds applications in various settings, including intensive care and emergency departments. Proficiency in this technique enables rapid diagnosis of respiratory muscle dysfunction in critically ill patients and those with unexplained dyspnea [135]. It can also aid in assessing patient-ventilator interaction and weaning failure. Two ultrasound approaches—mid-axillary intercostal and subcostal—are used to visualize the diaphragm [135]. With its wide availability and feasibility, respiratory muscle ultrasound, when combined with cardiac and lung ultrasound, becomes the preferred imaging modality in intensive care unit patients [135, 137]. Mastery in this technique allows for a quick assessment of the global function of the respiratory muscle pump, particularly in diagnosing diaphragm weakness or paralysis [135, 136]. However, challenges exist, emphasizing the need

for a systematic ultrasound approach in clinical practice and research [135]. While diaphragm ultrasound appears reliable for assessing changes in diaphragm thickness, caution is required when comparing individual patient results due to small observer-dependent variations [135]. Accurate muscle thickness measurement depends on operator skills, ultrasound physics, and patient characteristics. Issues such as unclear surrounding membranes and insufficient ultrasound beam angulation may introduce measurement errors [135, 137, 138].

Another approach to identifying the presence of muscle damage that can accommodate the internal location of the respiratory muscles is the use of blood biomarkers. The presence or change in concentration of particular biomarkers following a task provides indirect evidence of structural damage/changes that are presumably due to the exercise that was performed. They are simple, inexpensive and are minimally invasive and provide a more attractive method for evaluating respiratory muscle damage. The above-mentioned Table 2.1 provides a list of the common biomarkers used to detect muscle damage.

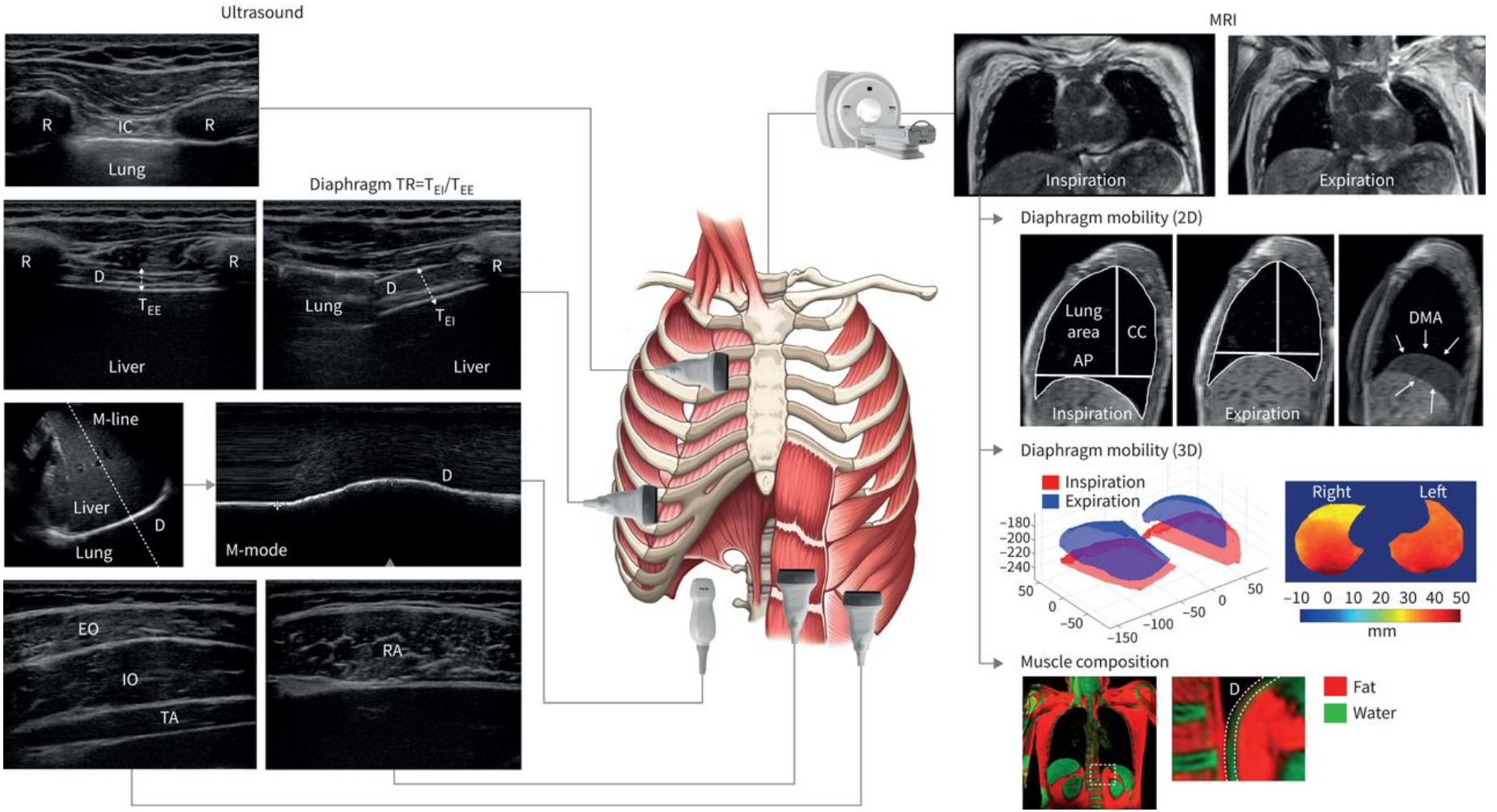

Figure 2.11 Overview of currently used respiratory muscle imaging techniques: ultrasound and magnetic resonance imaging (MRI). R: rib; IC: intercostal muscles; TR: thickening ratio; TEI: end-inspiratory thickness; TEE: end-expiratory thickness; D: diaphragm; EO: external oblique muscle; IO: internal oblique muscle; TA: transverse abdominal muscle; RA: rectus abdominis muscle; CC: cranio-caudal; AP: antero-posterior; DMA: diaphragmatic movement area [138].

2.6.6. The use of biomarkers to measure respiratory muscle damage

The use of biomarkers to indicate the presence of muscle damage in respiratory muscles is scarce with only two known studies using this technique. Mathur et al. [130] asked ten young and healthy participants to perform 60 min of ITL at 70% of their maximal inspiratory mouth pressure (P_{Imax}). P_{Imax} and maximal expiratory mouth pressures, delayed onset muscle soreness (DOMS) and plasma CK were measured prior to ITL, and at repeated time points after ITL (+4, +24 and +48 h). There was no change in P_{Imax}, maximal expiratory mouth pressures or CK following ITL despite the presence of DOMS. Foster et al. [33] evaluated the effects of ITL performed at 70% of P_{Imax} for 60 minutes and serum concentrations of both isoforms of sTnI (slow and fast), CK, DOMS, P_{Imax} and maximal expiratory mouth pressures were measured prior to and +1 h, three days and four days following ITL. There was an increase in fast sTnI identified +1 h and three days post-ITL while slow sTnI was elevated four days post-ITL. Other indices of respiratory muscle damage including CK and P_{Imax} and maximal expiratory mouth pressures did not change, however, DOMS was slightly but significantly increased following ITL [33]. Mathur et al. [130] and Foster et al. [33] did not use a sham or control ITL condition.

The findings from these studies [33, 130] indicate that the biomarker CK was not effective in indicating the presence of muscle damage in respiratory muscles, even though CK is commonly used for peripheral skeletal muscle damage detection [16]. This may be due to its large normal reference range for healthy participants. However, it could also be due to the type of task performed, as there was no change in P_{Imax} or maximal expiratory mouth pressures following 60 min of ITL, while the presence of DOMS was mixed between individuals. This may indicate that the qualitative level of muscle damage experienced was relatively low and that these measurements are not sensitive enough to accurately and consistently detect respiratory muscle damage. As such, using an alternative plasma biomarker that is readily detectable at low concentrations, such as sTnI, may provide a suitable tool for measuring muscle damage that is typically seen in the respiratory muscles [33].

2.7. Interventions to reduce respiratory muscle damage

Various strategies could be employed as preventive or therapeutic measures to address respiratory muscle damage and weakness in intensive care units (Figure

2.12) [76]. Preventive approaches in respiratory care involve several strategies. These include averting atrophy through lung and diaphragm protective ventilation, limiting the duration and degree of respiratory muscle inactivity, optimizing diaphragm effort and synchrony, avoiding injurious efforts, and preventing eccentric injury. These also include mitigating longitudinal atrophy, countering disuse atrophy with appropriate assist levels and effort, utilizing proportional modes, and avoiding myotoxic drugs [76]. On the therapeutic front, various interventions contribute to the management of respiratory muscle weakness. These include early whole-body mobilization, respiratory muscle endurance training, respiratory muscle strength training, IMT, and progressive threshold loading, which is tailored for specific populations on long-term ventilation. Additionally, efforts to restore progressive diaphragm function, supported by experimental and clinical data though not part of routine practice, and the use of electrical muscle stimulation, including phrenic nerve pacing, are integral components of therapeutic strategies [76]. These measures collectively aim to address and alleviate respiratory muscle weakness in the complex environment of the intensive care unit [76].

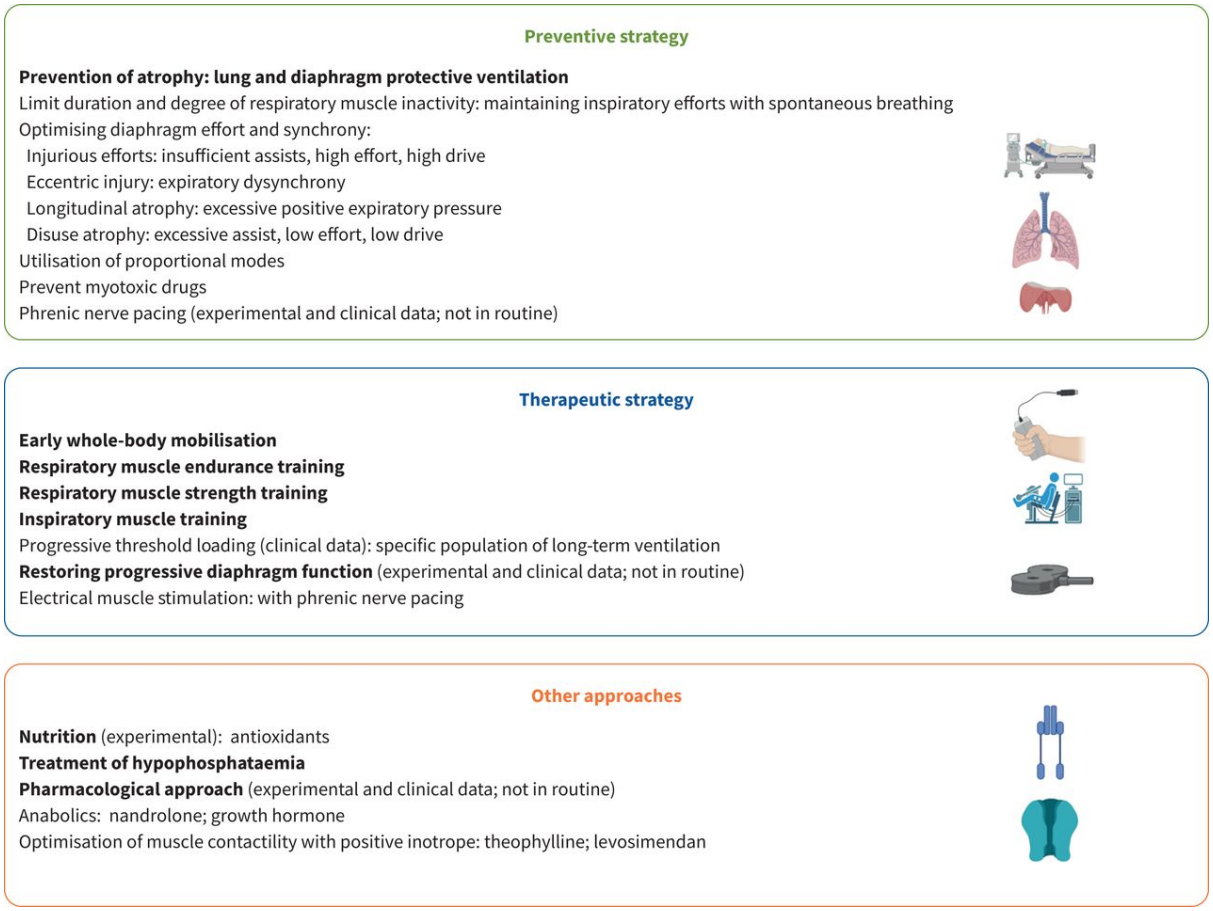

Figure 2.12 Interventions to manage respiratory muscle weakness in the intensive care unit [76].

2.7.1. Inspiratory muscle training

Several studies in both healthy individuals and COPD patients have demonstrated that repetitive respiratory muscle loading (i.e., respiratory muscle training) can increase the strength and endurance of the respiratory muscles and reduce respiratory muscle work [91, 139]. The most common form of respiratory muscle training is IMT. Pressure threshold IMT requires the participant to produce a negative pressure to overcome a threshold load and initiate inspiration, typically using a commercially available device with a spring-loaded poppet valve (Figure 2.13). A newer option is an electronic IMT device, which presents an innovative approach. These K-series devices are equipped with a distinctive electronically tapered flow resistive loading valve. This design ensures that resistance is continuously assessed and adjusted to align with decreasing strength throughout each breath, enabling a smoother flow and maximum volume. Unlike conventional pressure threshold devices that may cut off halfway through the breath, this technology results in a more satisfying and efficient breath,

delivering maximum flow, volume, and increased work per breath (https://www.powerbreathe.com/product-category/breathing-trainers/k-series-inspiratory-muscle-training-imt/). Training is normally undertaken twice daily involving 30 inspiratory breaths starting at 50% P_{Imax}. IMT has been reported to be effective in improving some components of pulmonary function, P_{Imax} and endurance and exercise capacity [140], and may reduce the vulnerability of respiratory muscles to exertion-induced damage.

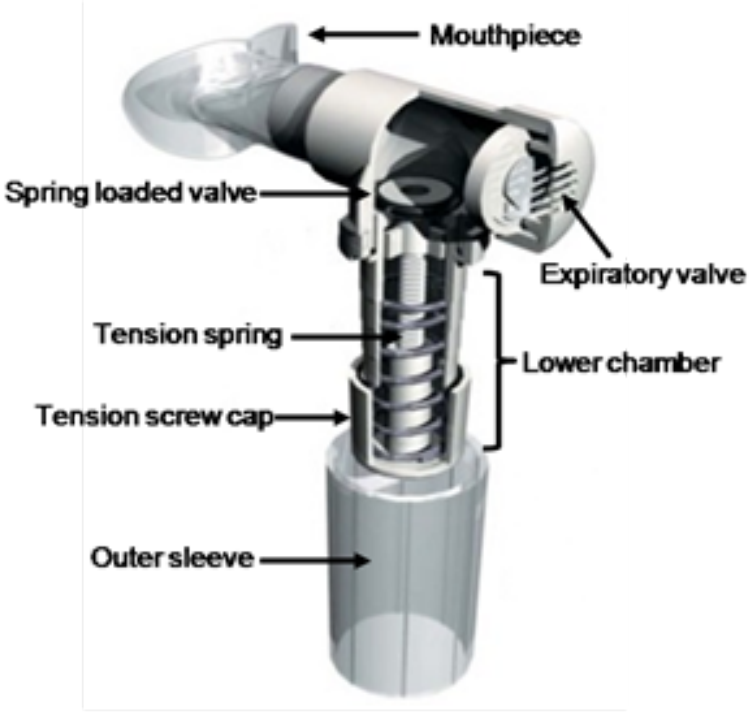

Figure 2.13 Example of commercially available pressure threshold inspiratory muscle training device. Adapted from www.powerbreathe.co.uk.

In healthy individuals, meta-analyses have demonstrated that IMT improves the strength and endurance of the inspiratory muscles and exercise performance [141]. Another study [142] systematically assessed the impact of IMT with POWERbreathe® on respiratory parameters and athletic performance in healthy, active adults. Out of 241 studies, 11 met the inclusion criteria for the systematic review, and nine were included in the meta-analysis. Results indicated that IMT led to significant improvements in P_{Imax} and substantial enhancements in forced vital capacity. Additionally, sports performance showed a significant improvement. The systematic

review concluded that IMT can enhance both respiratory function and athletic performance in physically active individuals [142].

In another meta-analysis [143], the effectiveness of physical therapy interventions, including conventional physical therapy, IMT, and early mobilization was compared on mechanical ventilation and weaning duration. A total of 18 randomized control trials (934 participants) were analyzed. Results showed that IMT and conventional physical therapy significantly reduced weaning duration, while early mobilization significantly reduced mechanical ventilation duration compared to conventional physical therapy alone. IMT + conventional physical therapy and early mobilization were identified as the most effective interventions for reducing weaning and mechanical ventilation durations, respectively. The review suggests recommending IMT or early mobilization to improve outcomes in mechanically ventilated patients, with caution due to heterogeneity [143].

Another meta-analysis analyzing the effects of IMT in COPD patients has demonstrated significant improvements in P_{Imax} and endurance [144], The same meta-analysis has also shown improvements in six- and 12-min walking distance and reductions in dyspnea assessed using a Borg score, transition dyspnea index and chronic respiratory questionnaire dyspnea score. Although the effect of IMT has already proved beneficial for other indices of respiratory performance, its effect in preventing or reducing respiratory muscle damage is unknown.

A recent systematic review [145] investigated the effects of IMT in individuals with obesity, focusing on respiratory and cardiovascular outcomes. Out of 705 initially identified studies, eight met eligibility criteria. IMT was found to significantly improve physical capacity (distance walked in the six-minute walk test) and P_{Imax} compared to controls. However, there were no significant changes in pulmonary function, body mass index, or metabolic parameters. The study suggests that IMT is beneficial for enhancing specific health aspects in individuals with obesity.

Training or rehabilitating muscles is a common practice among athletes, healthy individuals, and clinical populations, including those with COPD or those effected by COVID-19 [112, 146]. It has been suggested that respiratory muscles can undergo

training using the same principles applied to skeletal muscles, enhancing their strength, contraction speed, and endurance. Given that respiratory muscle weakness is a significant risk factor for unsuccessful weaning, IMT emerges as a promising strategy for mechanically ventilated patients facing prolonged weaning [112].

IMT serves different purposes, such as enhancing endurance or muscle strength, necessitating distinct strategies with varying physiological responses. The initiation of IMT can occur at different points, including 24 hours after the start of mechanical ventilation, upon the patient's awakening, during the transition to partially assisted ventilation, when the patient is deemed ready for weaning, or after a failed weaning attempt [76]. Several randomized studies have explored IMT in intensive care unit patients [147]. One study demonstrated that a 30% P_{Imax} inspiratory load for 5 minutes, twice a day for 7 days a week, increased P_{Imax} in the treatment group but had no impact on weaning success [148]. Another study [149] was conducted in patients ventilated for an average of 44 days. IMT was administered five days a week using the Threshold Inspiratory Muscle Trainer, providing inspiratory pressure loads ranging from -4 to -20 cmH_2O. Each day involved four sets of 6 to 10 breaths, with a two-minute rest period supported by mechanical ventilation between sets. The training device pressure was adjusted to the maximum level the patient could consistently open during inspiration, with daily progression as tolerated. Patients were directed to inspire and expire forcefully during the training sessions. Results showed that this IMT protocol improved P_{Imax} and increased the proportion of successful weaning [149].

A pilot controlled clinical study [112] was conducted to evaluate the impact of IMT on recovered COVID-19 patients who had undergone mechanical ventilation. Forty-two patients, aged 48 ± 9 years, were divided into an IMT group (undergoing two weeks of IMT) and a control group. Various parameters including lung function, dyspnea, quality of life, and six-minute walk test distance were assessed before and after the intervention. The IMT group showed significant improvements in forced vital capacity, forced expiratory volume in 1 s, quality of life and six-minute walk test distance, while the control group exhibited nonsignificant changes. The study concluded that a 2-week IMT program enhanced pulmonary function, functional performance, and quality of life and reduced dyspnea, in recovered COVID-19 patients who underwent mechanical

ventilation [112]. However, a placebo or sham IMT group was not used in this study which weakens internal validity and risks participant bias.

Another study [150] investigated the rehabilitative impact of IMT in individuals recovering from COVID-19 with prolonged symptoms. In this randomized trial with 281 participants, IMT did not significantly impact the primary outcome (Health-related quality-of-life and dyspnea questionnaires - King's Brief Interstitial Lung Disease - K-BILD) compared to a control group. However, IMT demonstrated clinically meaningful improvements in specific K-BILD domains related to dyspnea and chest symptoms. Additionally, improvements were observed in dyspnea according to the Transition Dyspnea Index, as well as in respiratory muscle strength and estimated aerobic fitness. The study suggests that IMT could be a valuable home-based rehabilitation strategy for individuals recovering from COVID-19, but individual responses may vary [150].

A randomized control trial [151] investigated the feasibility and safety of IMT during the acute phase of COVID-19 in hospitalized patients. Among 41 participants included in the analysis, IMT was completed successfully in 161 sessions among the 19 patients in the intervention group. Mortality totaled 2 in the control group and 3 in the intervention group and adverse events during intervention occurred in only 3 (1.8%) sessions, all of which were minor O_2 desaturations. Both groups showed improved P_{Imax}, reduced supplemental O_2 needs, and enhanced functional outcomes. Length of stay was shorter in the intervention group, and discharge disposition was similar between groups. The findings suggest that IMT is a feasible and safe intervention for hospitalized COVID-19 patients during the acute phase, supporting its potential application in acute care settings [151].

IMT may help to restore the muscle coordination lost during mechanical ventilation, by training the muscles to work together in a synchronized manner, resulting in a more enhanced respiratory muscle function [152, 153]. This may result in lower concentrations of biomarkers of muscle damage. However, whether IMT could reduce biomarkers of muscle damage and improve respiratory function and functional capacity in recovered COVID-19 patients after weaning from mechanical ventilation

was unknown. Whether it could prevent respiratory muscle damage by building up the strength and endurance of the respiratory muscles is yet to be determined.

2.8. Summary

Respiratory muscle damage may occur during and following excessive loading which exceeds the usual requirements of the muscle. Because of the essential role in ventilation, respiratory muscle damage may have serious consequences as it leads to respiratory muscle dysfunction. These may range from exercise intolerance and dyspnea through to ventilatory failure when the respiratory muscles can no longer provide enough ventilation to maintain arterial blood gases within a physiological range. The current techniques to measure respiratory muscle damage are muscle biopsies and imaging technologies, but they have problems because these are invasive and/or costly. Providing a simple and minimally invasive method to directly evaluate exertion induced respiratory muscle damage is important because it would be a relatively inexpensive, easy to perform tool to get quantitative and broadly accepted results.

Another approach to identifying the presence of muscle damage that can accommodate the internal location of the respiratory muscles is the use of blood biomarkers. The presence or change in concentration of particular biomarkers following a task provides indirect evidence of structural damage/changes that are presumably due to the exercise that was performed. They are simple, inexpensive and are minimally invasive and provide a more attractive method for evaluating respiratory muscle damage. Respiratory muscle work can be elevated at rest using ITL and VH and these techniques isolate the respiratory muscles in such a way, that any changes in systemic blood biomarkers can be assumed to originate from the respiratory muscles. The use of blood biomarkers to indicate the presence of muscle damage in respiratory muscles is scarce with only two known studies using ITL to increase respiratory muscle work. These studies only measured CK and TnI and there are other biomarkers that could be used to evaluate respiratory muscle damage. Another experimental approach that also allows respiratory muscles to be exercised without peripheral muscle involvement is VH performed at rest. This experimental technique results in a breathing and respiratory muscle recruitment pattern that is more ecologically relevant to exercise hyperpnea. However, the effects of VH on blood-

based biomarkers of respiratory muscle damage has not been tested. I used VH in this thesis as an experimental technique to elevate respiratory muscle work and to assess resultant damage with serum biomarkers.

COVID-19 can impair the respiratory muscles directly or through immune responses, leading to acute respiratory failure. Mechanical ventilation is crucial for severe cases but has both positive (improved oxygenation) and negative effects (complications like lung damage). Prolonged ventilation may cause respiratory muscle weakness and reduced function even after weaning. Managing these complexities is crucial for addressing the long-term consequences of COVID-19 on respiratory health. IMT shows promise in improving respiratory function and functional capacity for patients recovering from mechanical ventilation. IMT enhances muscle strength, endurance, and coordination, potentially reducing biomarkers of muscle damage. However, its specific effectiveness on biomarkers of respiratory muscle damage in recovered COVID-19 patients after weaning from mechanical ventilation remains unknown.

Accordingly, this research project undertook three research studies to determine a set of specific biomarkers of respiratory muscle damage in humans and evaluate the effect of IMT on biomarkers of respiratory muscle damage in recovered COVID-19 patients after weaning from mechanical ventilation.

CHAPTER 3: PAPER 1 – Biomarkers to measure respiratory muscle damage following inspiratory pressure threshold loading in healthy young men

3.1. Introduction

This paper is the first study of my PhD, and it describes how detecting respiratory muscle damage induced by whole-body exercise through the interpretation of blood biomarkers can be challenging due to the unidentifiable site of release. An experimental approach that allows the respiratory muscles to be exercised without peripheral muscle involvement is inspiratory pressure threshold loading (ITL). This study used this technique to increase inspiratory muscle work in resting healthy individuals, isolating the respiratory muscles and attributing any changes in systemic blood biomarkers to be originating from respiratory muscles. The aim of this study was to investigate the response of a panel of skeletal muscle damage biomarkers including fast and slow skeletal troponin I (sTnI), creatine kinase muscle-type (CKM), fatty acid-binding protein 3, myosin light chain 3 and myoglobin in response to ITL undertaken for 60 min on separate occasions at 70% (high ITL) and ~0% of maximal inspiratory mouth pressure (P_{Imax}) (Sham ITL) in healthy young men. We hypothesized that these biomarkers would increase following the 70% ITL condition compared to the Sham ITL condition. The main findings were that CKM (1 h and 24 h), fast sTnI (1 h) and slow sTnI (48 h) post 70% ITL were higher compared to the same timepoints after Sham ITL. Furthermore, these same markers showed no changes following Sham ITL. These results suggest that respiratory muscle damage was present at 1, 24 and 48 h following 70% ITL based upon our serum CKM and fast sTnI and slow sTnI findings. This chapter concludes that CKM and fast sTnI could be used to assess respiratory muscle damage immediately (+1 h), and CKM and slow sTnI could be used to assess respiratory muscle damage 24 and 48 h following conditions that cause elevated inspiratory muscle work.

3.3. Links and implications

As discussed in the literature review section of this thesis, respiratory muscle damage may occur during and following excessive loading which exceeds the usual requirements of the muscle. Excessive loading can be categorized in two ways - overload or overactivity. Overload is a condition when the force requirement is higher than usual requirements. Overactivity, in contrast, can be defined as increased work when the firing of the motor neuron is increased beyond its normal physiological levels or duty cycles. ITL was used to overload the respiratory muscles in study 1 and this induced respiratory muscle damage. In respiratory diseases, the respiratory muscles can experience both overload and overactivity because each breath may require a higher inspiratory muscle force and a higher breathing frequency (i.e., number of respiratory muscle contractions per minute).

Another experimental approach that also allows the respiratory muscles to be exercised without peripheral muscle involvement is volitional hyperpnea (VH) performed at rest. This technique involves participants mimicking at rest the breathing pattern they adopted during a prior exercise bout. As such, this experimental technique results in a breathing and respiratory muscle recruitment pattern that is more ecologically relevant to exercise hyperpnea. However, the effects of VH on blood-based biomarkers of respiratory muscle damage has not been tested. Accordingly, study 2 was designed to test if experimentally induced overactivity of the respiratory muscles via VH can induce respiratory muscle damage and if this can be detected in a panel of serum biomarkers.

CHAPTER 4: PAPER 2 – The effects of volitional hyperpnea on biomarkers of respiratory muscle damage in healthy young men

4.1. Introduction

Chapter 4 describes the second study of this PhD thesis and volitional hyperpnea (VH) was used to allow the respiratory muscles to be exercised without peripheral muscle involvement at rest. This is a more ecologically relevant way to test whether respiratory muscle damage occurs following high intensity exercise hyperpnea. The VH challenge mimicked the breathing (tidal volume, breathing frequency and duty cycle) and diaphragm recruitment (transdiaphragmatic pressures) patterns in a square wave manner to a level equivalent to those at 85% of participants maximum minute ventilation produced during a maximal incremental cycling test. In this chapter, the response of a panel of serum biomarkers, consisting of creatine kinase muscle type (CKM), and fast and slow skeletal troponin I (sTnI) was used to detect the presence of respiratory muscle damage in response to VH and control trials undertaken on separate occasions in healthy young men. This chapter concluded that that only slow sTnI was higher at +24 h post VH compared to the same timepoint after control trial. CKM and fast sTnI did not increase after VH compared with the control trial. These results suggest that respiratory muscle damage was present at +24 h following VH based upon the serum slow sTnI findings, but not evidenced by the serum CKM and fast sTnI findings.

4.2. Submitted paper

The effects of volitional hyperpnea on biomarkers of respiratory muscle damage in healthy young men

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Running Head: Biomarkers to measure respiratory muscle damage after volitional hyperpnea

Keywords: Biomarkers; Respiratory muscle damage; Volitional hyperpnea; Skeletal troponin I

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ABSTRACT

High-intensity exercise hyperpnea places substantial demands upon the respiratory muscles, but whether this causes respiratory muscle damage is unknown. We investigated respiratory muscle damage following volitional hyperpnea using a skeletal muscle damage biomarker panel. Eight healthy men (33 ± 2 years) underwent 10 min trials of volitional hyperpnea and rest (control) two weeks apart. Volitional hyperpnea consisted of mimicking the breathing (tidal volume, breathing frequency and duty cycle) and diaphragm recruitment (transdiaphragmatic pressure) patterns in a square wave manner to a level equivalent to those at 85% of participants maximum minute ventilation produced during a maximal incremental cycling test. Serum was collected before and at 1, 24, and 48 h after both volitional hyperpnea and control trials. Creatine kinase muscle-type (CKM), fast skeletal troponin I (sTnI) and slow sTnI were measured using enzyme-linked immunosorbent assay. Two-way analysis of variance revealed time x trial interaction effects for slow sTnI ($P = 0.018$), but not for CKM ($P = 0.072$) and fast sTnI ($P = 0.140$). Slow sTnI was significantly higher at +24 h post volitional hyperpnea ($P < 0.001$) as compared to same time point of the control trial. These results indicate that high-intensity exercise hyperpnea may induce a small amount of respiratory muscle damage as evidenced by the increases in slow sTnI. Future studies including more timepoints, different respiratory muscle exercise protocols and examining the differences between sexes could provide additional insights into the utility of blood biomarkers for identifying respiratory muscle damage.

INTRODUCTION

High-intensity exercise hyperpnea places substantial demands upon the respiratory muscles as breathing frequency, tidal volume and the work of breathing increase (1). Prolonged and strenuous exercise hyperpnea can also result in diaphragm fatigue (2). In peripheral limbs, skeletal muscle damage can also occur following unaccustomed, high-intensity exercise (3). However, there is limited information available about respiratory muscle damage compared to peripheral skeletal muscle damage (4, 5). One of the main factors contributing to this is the challenges associated with certain methodologies that are often employed to analyze peripheral muscle damage. Muscle biopsies, for example, can directly measure the extent of damaged fibers in peripheral skeletal muscle, but this technique has only been applied for the respiratory muscles with small tissue samples collected from patients having abdominal or thoracic surgery (6, 7). Although this method provides definitive evidence of damage to the muscle's structure, biopsies are invasive and difficult to sample on most respiratory muscles because of their internal location (8). One potential method for identifying damage to muscles without the need for invasive procedures involves the use of several imaging techniques, such as computed tomography scans, magnetic resonance imaging, and magnetic resonance spectroscopy. However, the utilization of these imaging techniques is accompanied with high costs, limited accessibility, and results that may also vary depending upon the particular technique used (9, 10). An alternative method for detecting muscular damage in the respiratory muscles is by the utilization of blood-based biomarkers (11). Indirect evidence of structural damage, potentially caused by exercise or overactivity of the respiratory muscles in specific respiratory disorders, could be inferred from the presence or increase in the concentration of blood-based biomarkers after exercise or respiratory muscle work (5, 12). Utilizing blood markers is an easy, cost-effective, and less invasive procedure compared with anatomical

pathology examination that may offer a more efficient and advantageous approach to detecting respiratory muscle damage (5, 11).

Iqbal et al. (12) and Foster et al. (11) have found that blood-based biomarkers of respiratory muscle damage increase following inspiratory pressure-threshold loading (ITL) in healthy young men. Foster et al. (11) measured skeletal troponin-I (sTnI) following 60 min of ITL. sTnI is a regulatory protein that plays an essential role in the contraction of skeletal muscles (13, 14). sTnI is an ideal biomarker for muscle damage due to its specificity to skeletal muscle tissue, quantitative measurability, and sensitivity, making it an accurate indicator of the extent of muscle damage when detected in the bloodstream (12, 15, 16). Foster et al. (11) observed that fast sTnI increased at 1 h (+24%) and 3 days (+72%) post ITL. Slow sTnI was elevated by 24% 4 days post ITL, but there was no change in creatine kinase. Iqbal et al. (12) observed that creatine kinase muscle-type (CKM) and fast sTnI increased immediately (+1 h) following ITL, while CKM and slow sTnI increased at +24 and +48 h (12). This finding suggests that CKM and fast sTnI could be used to assess respiratory muscle damage immediately, while CKM and slow sTnI could be used to assess respiratory muscle damage in the days following conditions that elevate inspiratory muscle work (12).

We used ITL as a tightly controlled experimental approach that allowed both primary and accessory respiratory muscles to be exercised without peripheral muscle involvement. This technique elevates inspiratory muscle work in individuals while at rest and thus isolates the respiratory muscles in such a way that any changes in systemic blood biomarkers can be assumed to originate from the respiratory muscles. Another experimental approach that also allows respiratory muscles to be exercised without peripheral muscle involvement is volitional hyperpnea performed at rest (17). This technique involves participants mimicking at rest the

breathing pattern they adopted during a prior exercise bout. As such, this experimental technique results in a breathing and respiratory muscle recruitment pattern that is more ecologically relevant to exercise hyperpnea. However, the effects of volitional hyperpnea on blood-based biomarkers of respiratory muscle damage has not been tested.

Accordingly, the aim of this study was to investigate the effects of volitional hyperpnea on blood-based biomarkers of respiratory muscle damage including CKM, and fast and slow sTnI. We hypothesized that CKM, fast and slow sTnI would increase following volitional hyperpnea compared to a control trial.

METHODS

Participants

Eight apparently healthy young men with respiratory function within normal limits volunteered to participate in the study (Table 1). The exclusion criteria were current cigarette smokers; history or current symptoms of cardiopulmonary disease; contraindications to exercise testing; and a body mass index of <18.5 or >30 kg/m^2. A self-reporting medical history questionnaire confirmed that participants were free from illness and injury and not taking any medication and/or dietary supplements during the study. All participants provided written, informed consent. All study procedures were approved by the University of Southern Queensland Human Research Ethics Committee (H20REA151), which adheres to the Declaration of Helsinki with the exception of registration in a database.

131 Table 1. Participant anthropometrics, respiratory function and maximal exercise capacity.
132 Values are mean ± SD.

Age (years)	33 ± 2
Height (cm)	176 ± 6
Body mass (kg)	83 ± 9
Body mass index (kg/m^2)	26.5 ± 2.5
FVC (L)	4.63 ± 0.88
FVC (% predicted)	92 ± 17
FEV_1 (L)	3.69 ± 0.44
FEV_1 (% predicted)	89 ± 12
FEV_1/FVC (%)	74 ± 7
FEV_1/FVC (% predicted)	98 ± 9
P_{Imax} (cmH_2O)	113 ± 4
P_{Imax} (% predicted)	103 ± 13
P_{dimax} (cmH_2O)	72 ± 20
$\dot{V}O_{2max}$ (mL/kg/min)	45 ± 9
$\dot{V}_{Emax}$ (L/min)	140 ± 4

133
134 FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; P_{Imax}, maximal inspiratory
135 mouth pressure; P_{dimax}, maximal transdiaphragmatic pressure; $\dot{V}O_{2max,}$ maximal oxygen
136 uptake; $\dot{V}_{Emax,}$ maximal minute ventilation. Predicted values for pulmonary volumes and
137 capacities are from Quanjer et al. (18) and for P_{Imax} are from Wilson et al. (19).
138
139

140 ***Experimental design***

141 Participants attended the laboratory for eight visits on separate days (Figure 1). During Visit 1,
142 height, body mass, pulmonary function and maximal inspiratory mouth pressure were assessed
143 according to published guidelines and statements (20, 21). Participants were also familiarized
144 with all the study procedures. During Visit 2, participants performed a maximal incremental
145 cycling test. During Visits 3 and 6, participants randomly performed either 10 min of volitional
146 hyperpnea whilst seated on a cycle ergometer at rest or adopted the same position seated on the
147 cycle ergometer and did not perform volitional hyperpnea (control). Blood samples were
148 collected from participants and stored at -80°C for subsequent analysis of respiratory muscle
149 damage biomarkers at rest and at +1 h, +24 h (Visit 4 and 7), and +48 h (Visit 5 and 8) post-
150 volitional hyperpnea and control trials. Visits 2 (maximal incremental cycling test), 5 (+48 h
151 after volitional hyperpnea or control) and 6 (volitional hyperpnea or control) were separated

by at least one week. To avoid the effects of the proceeding trial, a gap of at least one week was provided between the maximal incremental cycling test (Visit 2) and the first experimental condition (either volitional hyperpnea or control; Visit 3). In addition, a gap of one week was provided between the day of the last blood sample for the first experimental condition (+48h; Visit 5) and the second experimental condition (either volitional hyperpnea or control; Visit 6). Participants were required to refrain from moderate-vigorous exercise for at least two days prior to Visit 2, 3 and 6 and to not exercise in the 48 h after each visit. Participants were also instructed to abstain from food (4 h), caffeine (12 h), and alcohol (24 h) before laboratory visits.

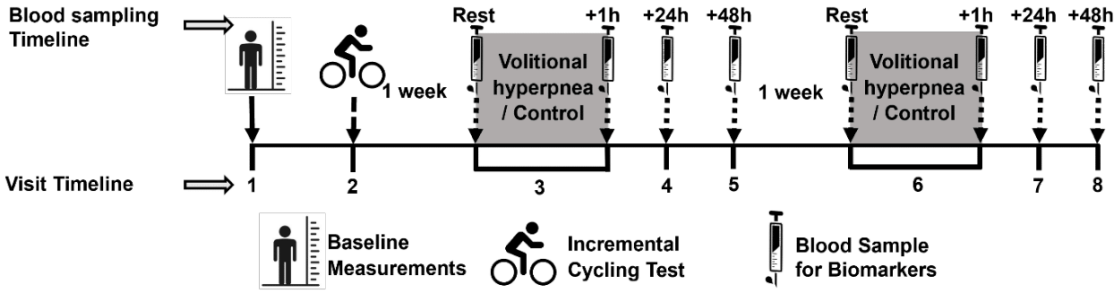

Figure 1: Schematic of experimental design.

Anthropometrical measures and respiratory function

Height and body mass were recorded using a wall mounted electronic stadiometer (Seca 213; Seca, Hamburg, Germany) and an electronic scale (Tanita BC-541; Tanita, Kewdale, Australia), respectively. Pulmonary function was assessed using a spirometer (JAEGER® Vyntus; CareFusion, San Diego, CA, USA) according to published guidelines (20). A hand-held mouth pressure meter (MicroRPM; CareFusion, Frenchs Forest, Australia) was used to measure maximal inspiratory mouth pressure as an index of global inspiratory muscle strength. The maneuver was performed while seated, initiated from residual volume sustained for a

minimum of 1 s. The maneuver was repeated until three sequential measurements differing by no more than ± 10% or ± 10 cmH_2O, whichever is smallest. The greatest value is recorded for later analysis (17).

Maximal incremental cycling test

Participants undertook a maximal incremental exercise cycling test to exhaustion on an electronically braked cycle ergometer (Corival; Lode, Groningen, Netherlands). Each test began with 5 min of rest and cycling then began at 0 W and power was subsequently increased by 20 W every 30 s in order to result in exercise intolerance within 8-12 min. Participants maintained a constant self-selected cadence above 60 revs/min. Exercise ceased at the limit of tolerance or when cycling cadence could not be maintained above 60 revs/min. Participants wore a facemask (Model 7940; Hans Rudolph, Shawnee Mission, KS, USA) which was tightly fitted to minimize leaks and connected to a turbine flow sensor (Digital volume transducer; Vyaire Medical, Chicago, IL, USA) that was calibrated using a 3 L syringe. Pulmonary gas exchange was measured breath by breath using a metabolic cart (Vmax® Encore PFT system; Vyaire Medical, Chicago, IL, USA). The highest oxygen uptake ($\dot{V}O_2$) and minute ventilation ($\dot{V}_E$) recorded in any 30 s period was defined as $\dot{V}O_{2max}$ and $\dot{V}_{Emax}$, respectively.

Volitional hyperpnea

Participants performed volitional hyperpnea at rest whilst seated on the cycle ergometer in a body position identical to that adopted during the maximal incremental cycling test. Participants mimicked the breathing (tidal volume, breathing frequency and duty cycle) and diaphragm recruitment (transdiaphragmatic pressure; P_{di}) patterns in a square wave manner to

195 a level equivalent to those at 85% of their $\dot{V}_{Emax}$ produced during the maximal incremental
196 cycling test. Our pilot work and others showed that this was the maximal exercise breathing
197 pattern that could be maintained for 10 min (22). An audio metronome paced breathing
198 frequency and duty cycle and real-time visual feedback of tidal volume and P_{di} was provided
199 throughout the test. Isocapnia was maintained during volitional hyperpnea by adding carbon
200 dioxide into the inspiratory circuit in order to maintain resting arterial carbon dioxide partial
201 pressures.

202

203 ***Respiratory pressures***

204 Respiratory muscle work was quantified by measuring esophageal pressure (P_e) and gastric
205 pressure using two 10 cm balloon-tipped latex catheters (Model 47-9005; Ackrad Laboratories,
206 Cranford, NJ) attached with two different pressure transducers (MLT844; AD Instruments,
207 Dunedin, New Zealand). Co-Phenylcaine Forte Spray (Lignocaine Hydrochloride 5%;
208 Phenylephrine Hydrochloride 0.5%; ENT Technologies Pty Ltd, Hawthorne, Australia) was
209 used to initially anesthetize the nasal passage. Then, catheters were passed peri-nasally into the
210 lower third of the esophagus and stomach, respectively. During the first experimental trial, the
211 distance from the tip of the nares to the most distal point of the catheters was recorded and
212 replicated in the subsequent trial. The esophageal and gastric balloons were filled with 1 and 2
213 mL of air, respectively. The position of the balloons was confirmed with repeated sniffs until
214 a positive deflection in gastric pressure was observed for the gastric catheter, and the
215 esophageal catheter was withdrawn until a negative deflection in P_e was observed. An
216 occlusion test was then performed to confirm the catheters location in the esophagus (23). P_{di}
217 was calculated online by subtracting P_e from gastric pressure. P_{di} and P_e were integrated over
218 the period of inspiratory flow and multiplied by breathing frequency and labelled the

219 diaphragm pressure-time product (PTP_{di}) and the inspiratory muscle pressure-time product
220 (PTP_e), respectively. P_{di} and P_e were also normalized using the maximum pressure recorded
221 during any maximal inspiratory capacity maneuver performed at rest or during the volitional
222 hyperpnea or control trials for a given experimental visit.

223

224 ***Ventilatory, cardiorespiratory and perceptual responses***

225 Ventilatory responses during volitional hyperpnea and control trials were measured using a
226 pneumotach (Model 3813; Hans Rudolph, Shawnee Mission, KS, USA) inserted into the mouth
227 port of the two-way non-rebreathing valve. Volume was obtained by numerical integration of
228 the flow signal. Operational lung volumes were quantified by measuring inspiratory capacity
229 relative to forced vital capacity. During the resting stage and post-volitional hyperpnea and
230 control trials, participants performed forced vital capacity and inspiratory capacity maneuvers
231 in triplicate (24). Participants performed further inspiratory capacity maneuvers in duplicate
232 every 2 min (i.e., in the middle of the 2^{nd}, 4^{th}, 6^{th}, 8^{th} and 10^{th} min) during the volitional
233 hyperpnea and control trials. Strong verbal encouragement was given during each maximal
234 inspiratory effort maneuver. During this time, participants were asked to look forward,
235 minimize any head or neck movement, keep a loose grip on the handlebars, and to avoid talking
236 or swallowing. To confirm that a maximal inspiratory effort was made, we verified that peak
237 inspiratory P_e during each inspiratory capacity maneuver matched that obtained at rest. End-
238 tidal partial pressure of carbon dioxide was measured via the expiratory port of the two-way
239 nonrebreathing valve, which was connected to a gas analyzer (ML206; AD Instruments, Bella
240 Vista, Australia). Heart rate was measured using the R-R interval for a three lead ECG (AD
241 Instruments, Bella Vista, Australia) and estimated arterial oxygen saturation was measured
242 using a pulse oximeter (Radical-7 Pulse CO-Oximeter; Masimo Corporation, Irvine, CA,

243 USA), respectively. Breathing discomfort, defined as "a feeling of labored or difficult
244 breathing" was measured using the modified 0-10 category ratio Borg Scale (25) as a measure
245 of dyspnea intensity (26), during the final min of rest and after every 2 min after the inspiratory
246 capacity maneuvers during volitional hyperpnea and control trials (i.e., at the end of 2^{nd}, 4^{th},
247 6^{th}, 8^{th} and 10^{th} min).

248

249 ***Data capture and analysis***

250 Raw data were sampled using a 16-channel analogue-to-digital data acquisition system
251 (PowerLab 16/35; AD Instruments, Bella Vista, Australia) at 200 Hz. Data was recorded using
252 LabChart v8.1.2 software (AD Instruments, Bella Vista, Australia). Non-physiological data
253 that resulted from swallowing, coughing, and breath holding were identified by visual
254 inspection and removed. Respiratory muscle pressure, ventilatory and cardiorespiratory data
255 were continuously sampled and were analyzed in 1 min epochs. These were in the final min of
256 rest and the 2^{nd} (1-2 min), 4^{th} (3-4 min), 6^{th} (5-6 min), 8^{th} (7-8 min) and 10^{th} (9-10 min) min of
257 volitional hyperpnea and control trials.

258

259 ***Blood sampling and enzyme-linked immunosorbent assays***

260 Ten mL of venous blood was sampled and collected at each time point from an antecubital vein
261 via a BD Vacutainer Winged Blood Collection Set (BD Vacutainer® Safety-Lok™; Franklin
262 Lakes, NJ, USA) into serum separator tubes (BD Vacutainer® SST™ Tubes; Franklin Lakes,
263 NJ, USA). Samples were centrifuged at 2500 rpm for 10 min. The serum was then aliquoted
264 and stored at -80°C until biochemical assays were performed. Enzyme-linked immunosorbent
265 assays (ELISA) were performed for serum biomarkers using commercially available kits:
266 Human CKM (Catalog No. ab185988 Abcam, Cambridge, UK); Human Troponin I Type 2,

Fast sTnI (Catalog No. RK02421 Abclonal, Woburn MA, USA); and Human Troponin I Type 1, Slow sTnI (Catalog No. RK02420 Abclonal, Woburn MA, USA). ELISA were performed by following manufacturers' instructions for each specific kit. The assays had detection limits of 30 U/ml (CKM), 17.59 pg/mL (Slow sTnI) and 30 pg/mL (Fast sTnI). To minimize the effect of inter-assay variation, markers from both volitional hyperpnea and control trials were measured using the same assay plate.

Statistical analysis

Statistical analyses were performed using SPSS 25 for Windows (IBM, Chicago, IL, USA). An initial power calculation was performed on the basis of our previous work (12), showing that six participants would be required to demonstrate a 10% increase in sTnI with an alpha of 0.05. Normality of the data was assessed by visual inspection of histograms. The data from the volitional hyperpnea and control trials were analyzed using a two-way analysis of variance (ANOVA) to determine the effects of 'time' (rest, 2, 4, 6, 8 and 10 min) for ventilatory, cardiorespiratory, perceptual and pressure responses or blood analyses (baseline, +1 h, +24 h and +48 h) and 'trial' (volitional hyperpnea vs. control). Following significant interaction effects, pairwise comparisons were made using the Bonferroni method (adjustments for multiple comparisons). Statistical significance was set at $P < 0.05$. Results are presented as means ± SD.

RESULTS

Ventilatory, cardiorespiratory, perceptual and pressure responses

The ventilatory, cardiorespiratory, perceptual and pressure responses at rest and during the volitional hyperpnea and control trials are shown in Table 2 and Figures 2 and 3 along with the

main and interaction effects. Significant time x trial interaction effects ($P < 0.05$) were found for minute ventilation, duty cycle, absolute end-inspiratory lung volume and as a percentage of forced vital capacity, heart rate, rating of perceived dyspnea and P_e and P_{di} as a percentage of maximum. These responses were significantly higher during volitional hyperpnea compared to the control trial (all $P < 0.05$; Table 2). Time x trial interaction effects ($P < 0.05$) were also observed for peak P_{di}, breathing frequency and tidal volume. These responses were significantly higher at each time point during volitional hyperpnea compared to the control trial (all $P < 0.05$; Figure 2). Time x trial interaction effects ($P < 0.05$) were also observed for PTP_{di} and PTP_e, but not for PTP_{di}/PTP_e. These responses were significantly higher during volitional hyperpnea compared to the control trial (all $P < 0.05$; Figure 3). Significant time x trial ($P = 0.01$) interaction effects were found for maximal P_{di} measured during the maximal inspiratory capacity maneuvers before and at the end of the volitional hyperpnea and control trials. There was a decrease in maximal P_{di} during the volitional hyperpnea trial (pre: 72.1 ± 20.0 vs. post: 52.1 ± 21.7 cmH_2O, $P = 0.05$) while no changes were observed in maximal P_{di} before and after the control trial (pre: 70.1 ± 5.9 vs. post 71.4 ± 9.9 cmH_2O, $P = 0.63$). These values provide an indirect evidence of diaphragm fatigue after the volitional hyperpnea trial. There were no time x trial interaction effects for absolute end-expiratory lung volume and as a percentage of forced vital capacity, partial pressure of end-tidal carbon dioxide and estimated arterial oxygen saturation (Table 2).

Respiratory muscle damage biomarkers

The respiratory muscle damage biomarker responses at rest and after volitional hyperpnea and control trials are shown in Figure 4. There was a time x trial interaction effect for slow sTnI only ($P = 0.018$), which was higher compared to the control trial at +24 h ($P < 0.01$) post-

315 volitional hyperpnea. There were no differences observed at +1 h and +48 h post-volitional
316 hyperpnea compared with the control trial for slow sTnI. There were no time x trial interaction
317 effects for CKM (P = 0.072) and fast sTnI (P = 0.14).

318 Table 2. Ventilatory, cardiorespiratory, perceptual and pressure responses to the volitional hyperpnea and control trials. Values are mean ± SD.

Variable	Trial	Rest	2 min	4 min	6 min	8 min	10 min	Time	Trial	Time x Trial
$\dot{V}_E$ (L/min)	VH	14.3 ± 4.8	119.6 ± 32.6*	118.3 ± 25.2*	116.2 ± 24*	130.5 ± 30.9*	123 ± 36*	< 0.001	< 0.001	< 0.001
	Control	16.6 ± 2.8	16.0 ± 2.5	16.6 ± 3.0	13.6 ± 2.9	16.4 ± 3	15.1 ± 3.1			
Ti/ToT	VH	0.39 ± 0.05	0.50 ± 0.04*	0.50 ± 4*	0.49 ± 0.04*	0.47 ± 0.4*	0.45 ± 0.05*	< 0.001	0.015	< 0.001
	Control	0.41 ± 0.05	0.41 ± 0.04	0.41 ± 5	0.40 ± 0.05	0.42 ± 0.5	0.41 ± 0.04			
EILV (L)	VH	4.6 ± 0.9	4.9 ± 0.9	4.8 ± 0.9*	5.4 ± 1*	5.8 ± 0.5*	5.6 ± 0.8*	< 0.001	0.009	< 0.001
	Control	4.6 ± 0.5	4.7 ± 4	4.2 ± 0.7	4.0 ± 0.8	4.3 ± 0.7	4.1 ± 0.6			
EILV (%FVC)	VH	69 ± 11	95 ± 12*	93 ± 10*	94 ± 13*	95 ± 15*	89 ± 14*	< 0.001	0.002	< 0.001
	Control	69 ± 10	70 ± 12	68 ± 12	67 ±13	71 ± 12	69 ± 10			
EELV (L)	VH	2.7 ± 0.7	3.4 ± 0.3	3.1 ± 0.3	3.4 ± 0.3	3.0 ± 0.3	3.0 ± 0.4	0.314	0.508	0.117
	Control	3.5 ± 0.4	3.6 ± 0.3	3.2 ± 0.6	2.9 ± 0.8	3.2 ± 0.6	3.1 ± 0.7			
EELV (%FVC)	VH	52 ± 9	55 ± 7	53 ± 5	56 ± 6	53 ± 5	54 ± 7	0.556	0.313	0.237
	Control	60 ± 6	63 ± 6	58 ± 10	53 ± 13	57 ± 10	56 ± 12			
$P_{ET}CO_2$ (mmHg)	VH	35.3 ± 4.6	36.0 ± 3.2	37.1 ± 3.2	37.8 ± 3.1	38.5 ± 3.0	35.3 ± 4.4	0.021	0.814	0.070
	Control	37.0 ± 3.5	35.8 ± 3.0	37.4 ± 3.2	34.2 ± 4.5	37.8 ± 3.3	35.5 ± 2.8			
SaO_2 (%)	VH	97.1 ± 1.3	97.7 ± 1.4	97.0 ± 1.6	96.7 ± 1.6	96.0 ± 1.1	96.7 ± 1.3	0.835	1.000	0.089
	Control	96.7 ± 1.8	96.6 ± 1.4	97.7 ± 1.4	97.4 ± 1.3	96.6 ± 1.3	97.4 ± 1.3			
HR (beats/min)	VH	69 ± 11	87 ± 11*	83 ± 9*	82 ± 7*	85 ± 8*	77 ± 9*	< 0.001	0.070	< 0.001
	Control	70 ± 8	73 ± 9	71 ± 10	66 ± 11	74 ± 10	71 ± 10			
RPD	VH	0.4 ± 0.4	5.4 ± 0.6*	6.9 ± 1.1*	7.3 ± 1*	8.1 ± 1*	8.2 ± 1.7*	< 0.001	< 0.001	< 0.001
	Control	0.4 ± 0.4	0.6 ± 0.3	0.6 ± 0.2	0.4 ± 0.3	0.5 ± 0.3	0.4 ± 0.3			
P_e (% maximum)	VH	29 ± 7.5	78 ± 26*	79 ± 24*	79 ± 13*	72 ± 18*	75 ± 21*	< 0.001	< 0.001	< 0.001
	Control	35 ± 7.5	32 ± 6	37 ± 9	30 ± 7.8	36 ± 8.6	38 ± 8.5			
P_{di} (% maximum)	VH	31 ± 5	73 ± 11*	72 ± 12*	76 ± 9*	74 ± 10*	75 ± 11*	< 0.001	< 0.001	< 0.001
	Control	27 ± 7	27 ± 4	33 ± 4	26 ± 4	31 ± 3	31 ± 4			

319 $\dot{V}_E$, minute ventilation; VH, volitional hyperpnea; Ti/ToT, duty cycle; EILV, end-inspiratory lung volume; FVC, forced vital capacity; EELV, end-expiratory
320 lung volume; $P_{ET}CO_2$, partial pressure of end-tidal carbon dioxide; SaO_2, estimated arterial oxygen saturation; HR, heart rate; RPD, rating of perceived
321 dyspnea; P_e, esophageal pressure; P_{di}, transdiaphragmatic pressure; % maximum, the pressure swing as a % of maximum recorded pressure. *Significantly
322 different between volitional hyperpnea and control trials ($P < 0.05$). Note that P_e is a negative number, but for clarity it has been provided as a positive.

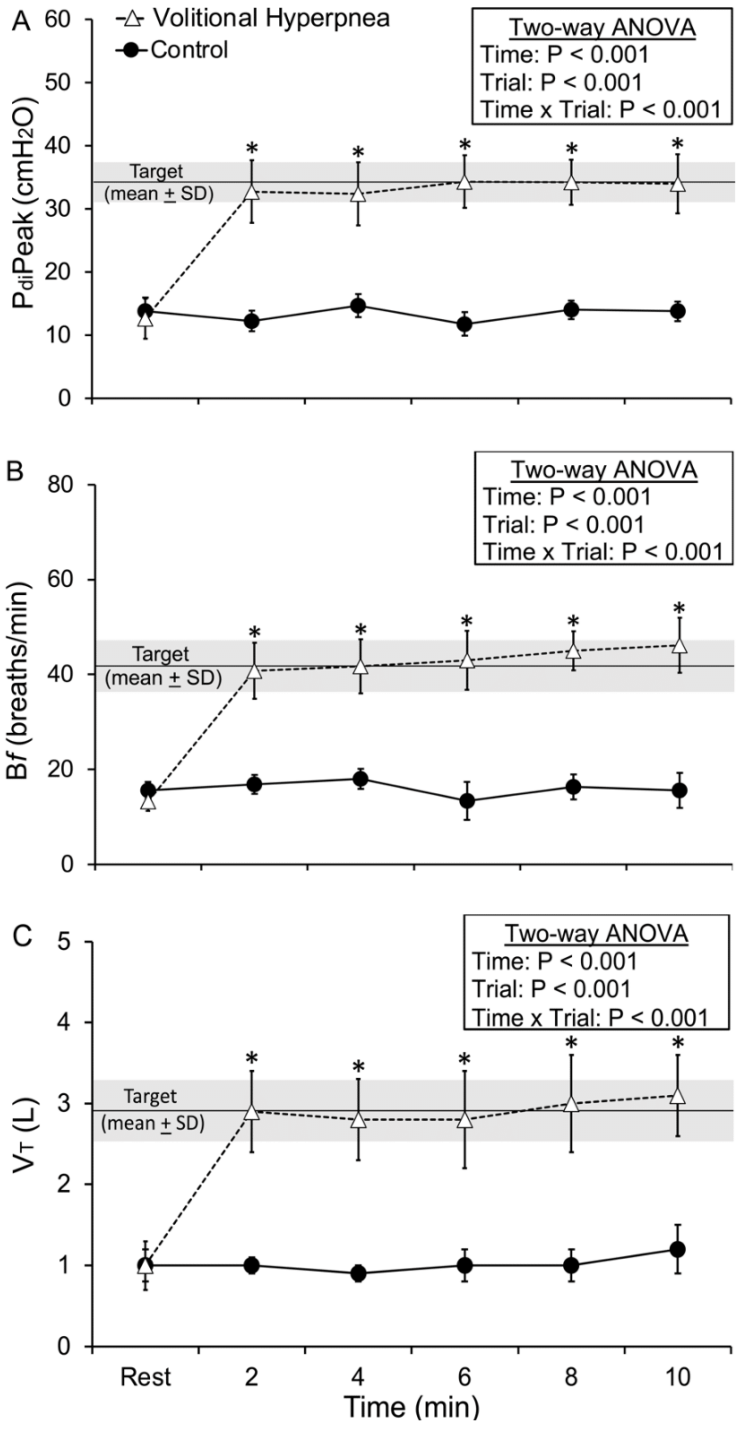

Figure 2. Peak transdiaphragmatic pressure ($P_{di}Peak$; A), breathing frequency ($B\dot{f}$; B), and tidal volume (V_T; C) responses to the volitional hyperpnea and control trials. Values are means ± SD. The horizontal black line denotes the mean volitional hyperpnea target and the shaded area the SD of the target. *Significantly different between volitional hyperpnea and control trials ($P < 0.05$; n = 8, males).

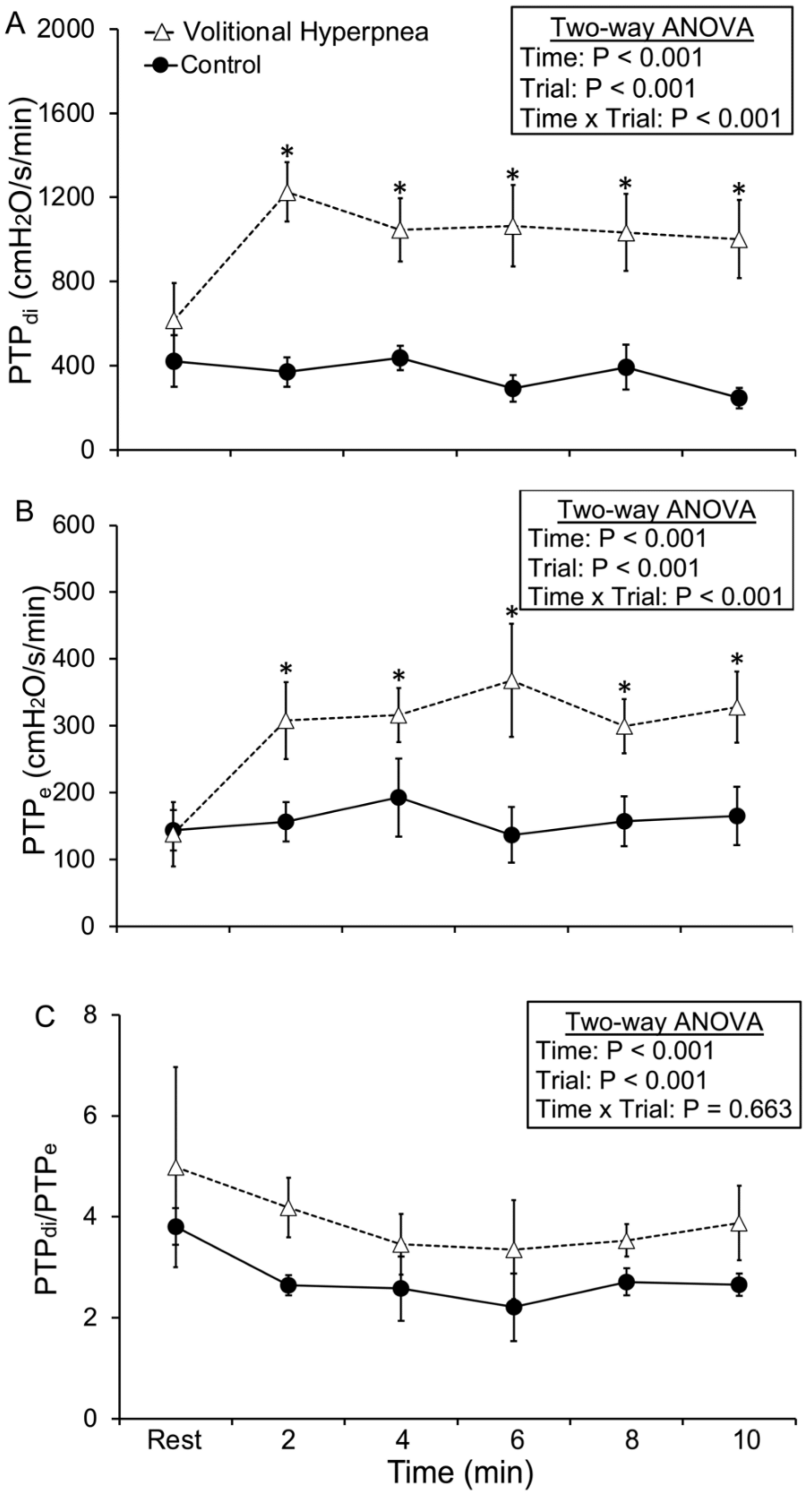

Figure 3. Transdiaphragmatic pressure time product (PTP_{di}; A), esophageal pressure time product (PTP_e; B) and the ratio between PTP_{di} and PTP_e (PTP_{di}/PTP_e; C) responses to the volitional hyperpnea and control trials. Values are means ± SD. *Significantly different between volitional hyperpnea and control trials ($P < 0.05$; n = 8, males).

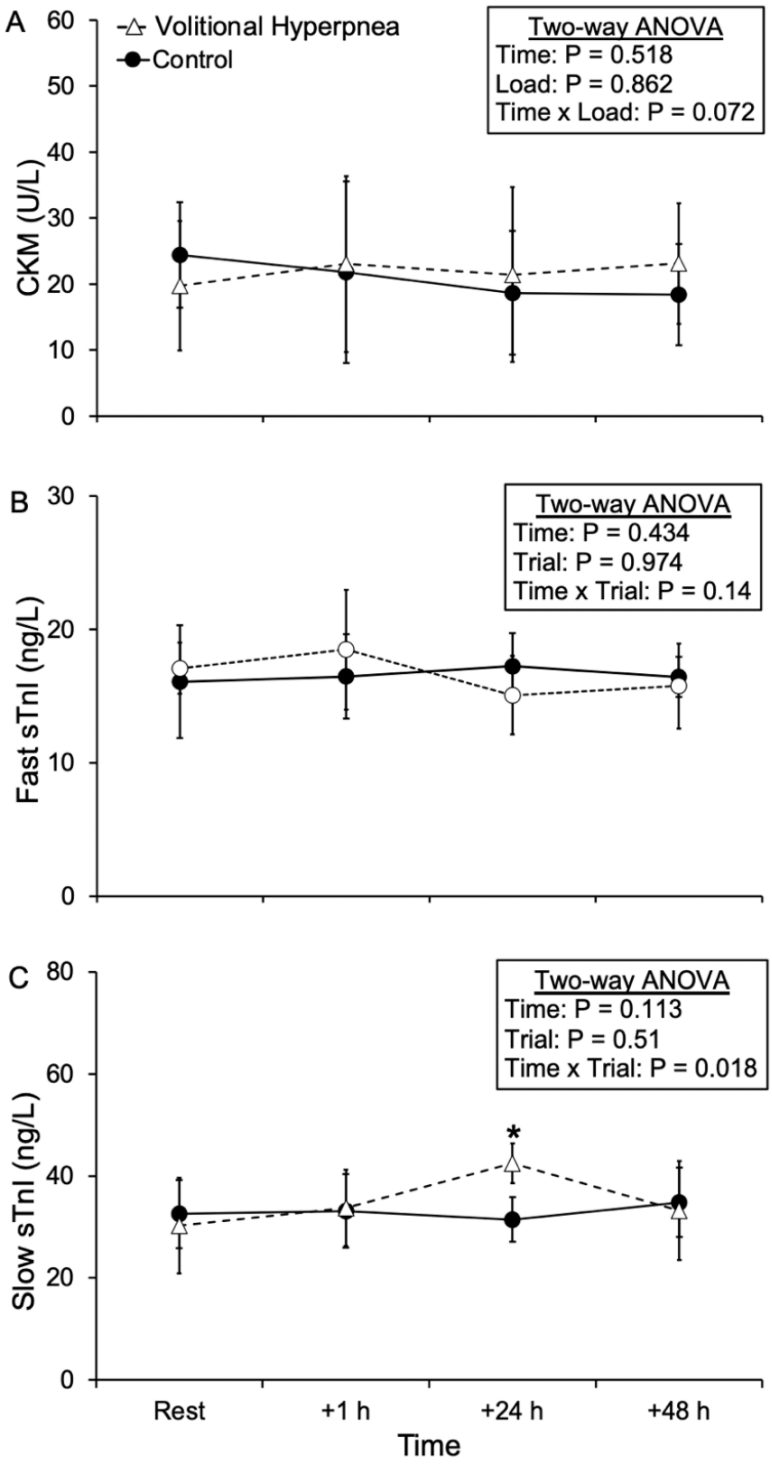

Figure 4. Creatine kinase muscle-type (CKM; A), fast skeletal troponin I (Fast sTnI; B), and slow skeletal troponin I (Slow sTnI; C) responses to the hyperpnea and control trials. Values are means ± SD. *Significantly different between the volitional hyperpnea and control trials ($P < 0.05$; n = 8, males).

DISCUSSION

Main findings

We investigated the response of a potential panel of serum biomarkers, consisting of CKM, and fast and slow sTnI to detect the potential presence of respiratory muscle damage in response to volitional hyperpnea and control trials undertaken on separate occasions in healthy young men. The main finding was that only slow sTnI was higher at +24 h post-volitional hyperpnea compared to the same timepoint after the control trial. CKM and fast sTnI did not increase after volitional hyperpnea compared with the control trial. These results suggest that respiratory muscle damage may be present at +24 h following volitional hyperpnea based upon our serum slow sTnI findings, but not evidenced by our serum CKM and fast sTnI findings. Our results of increased serum slow sTnI findings provide evidence of respiratory muscle damage.

Respiratory muscle damage biomarkers

To our knowledge, we are the first to report previously investigated and recommended respiratory muscle damage biomarkers, CKM, fast sTnI and slow sTnI in healthy young men in response to volitional hyperpnea that mimicked the breathing (tidal volume, breathing frequency and duty cycle) and diaphragm recruitment (P_{di}) patterns achieved during high-intensity exercise. sTnI is a regulatory protein that plays an essential role in the contraction of skeletal muscles (13, 14). We observed that slow sTnI was higher at +24 h post-volitional hyperpnea compared to the same timepoint after the control trial. These findings align with the outcomes of our previous study where healthy young men performed 60 min of ITL at a resistance equivalent to 70% of their maximal inspiratory mouth pressure and serum was

collected at the same time points as the present study (+1 h, + 24 h and +48 h) (12). We found in that study that slow sTnI increased at +24 h and +48 h post ITL and is also in agreement with Foster et al. (11) who found that slow sTnI was elevated by 24% 4 days post ITL also at a resistance equivalent to 70% of maximal inspiratory mouth pressure. sTnI exhibits the characteristics of an optimal indicator for skeletal muscle damage, including exclusive specificity to skeletal muscle, a wide diagnostic timeframe that enables early (within 1-6 hours after onset) and late (after 24-48 hours) detection, and a high level of sensitivity with a notable response magnitude (27). Our current study supports the conclusions made by Foster et al. (11) that sTnI has superior sensitivity compared to other non-specific biomarkers or indices of respiratory muscle damage.

We did not observe an increase in CKM and fast sTnI at any time point post-volitional hyperpnea compared to the control trial. This is in contrast to our previous finding using ITL (12), where we observed that CKM and fast sTnI increased within 1 hour of ITL and CKM was increased after 24 and 48 hours of ITL. The possible explanation for this finding may be the way the respiratory muscles are loaded during ITL and volitional hyperpnea. Firstly, while ITL principally loaded the inspiratory muscles, whereas volitional hyperpnea requires heightened recruitment of both inspiratory and expiratory muscles. Both primary and accessory muscles (of inspiration and expiration) underwent recruitment and overactivity during volitional hyperpnea, thus we can suspect all of these respiratory muscles as potential sources of respiratory muscle damage biomarkers in serum. Secondly, volitional hyperpnea requires more overactivity of the respiratory muscles rather than overload which is observed in ITL (see *mechanisms of respiratory muscle damage to volitional hyperpnea* below). Alternatively, our volitional hyperpnea protocol may not have been at a sufficient intensity and/or duration to cause respiratory muscle damage because the degree of exertion was lower compared with ITL.

Volitional hyperpnea may underestimate the respiratory muscle damage that may occur during whole-body exercise. For example, Babcock et al. (28) reported that the workload of the diaphragm needs to be 60-80% higher during volitional hyperpnea compared to whole-body exercise to achieve similar levels of diaphragm fatigue. Two potential contributors to diaphragm fatigue during high-intensity whole-body exercise are the elevated levels of circulating metabolites from fatiguing peripheral muscles and compromised blood flow to the respiratory muscles. Given that these two factors are absent during isolated overactivity of the respiratory muscles (i.e., during volitional hyperpnea), respiratory muscle damage might be less than what is expected during whole-body exercise. This possibly resulted in an insignificant increase in respiratory muscle damage biomarkers (fast sTnI and CKM) in our study. Another possible explanation may be that CKM and fast sTnI are not sensitive enough to detect the phases of damage caused by volitional hyperpnea as compared to slow sTnI. Further studies utilizing volitional hyperpnea at differing intensities and durations to induce isolated respiratory muscle damage could clarify the relative sensitivities of these serum biomarkers to measure respiratory muscle damage.

Different other biomarkers can also be utilized at various time points to detect respiratory muscle damage along with our recommended biomarkers in this study. Initially, assessments including diaphragmatic thickness via ultrasound (29) and maximal inspiratory mouth pressure (30) can reveal early signs of damage. Changes in chest wall motion (31) and blood gas analysis (32, 33) can also provide early indications. As time progresses, EMG can assess electrical activity changes (34), while inflammatory markers may rise, indicating the body's response to ongoing muscle damage (35). The collective use of these markers may detect the presence of

410 muscle damage and may relate to earlier and later events of respiratory muscle damage over
411 time.

412

413 ***Mechanisms of respiratory muscle damage to volitional hyperpnea***

414 Excessive loading that surpasses the normal demands of the respiratory muscles might lead to
415 muscular damage, both during and after the activity. Excessive loading can be classified into
416 two categories - overload or overactivity (36). Overload is a condition when the force
417 requirement is higher than usual while overactivity can be defined as an increased work rate
418 when motor neurons fire much higher than typically experienced during physiological duty
419 cycles (36). Overactivity occurs during many endurance exercise activities and in several
420 disease conditions such as asthma or chronic obstructive pulmonary disease (36). In respiratory
421 diseases the respiratory muscles can undergo both overload and overactivity due to the
422 increased demand for inspiratory muscle force and breathing frequency (36). In the present
423 study, volitional hyperpnea was used to induce a similar kind of overactivity of respiratory
424 muscles, by voluntarily increasing breathing frequency and tidal volume. This exceeded the
425 respiratory muscles usual capability, and this overactivity was aimed at causing damage to the
426 muscles involved in breathing (11, 30). Volitional hyperpnea induced respiratory muscle
427 damage may specifically affect some specific number of components in the muscle cells, or it
428 could cause minor tears in various components such as the sarcolemma, z-disk, basal lamina,
429 and surrounding connective tissues. Additionally, it can lead to damage in the cytoskeleton and
430 contractile elements (37-40). In the current study, we successfully detected a small but
431 significant increase in the concentrations of slow sTnI in the participants' serum, suggesting
432 the presence of respiratory muscle damage after volitional hyperpnea. Fast and slow sTnI
433 correspond to fast and slow twitch muscle fibers, respectively. The most accurate estimates of

fiber type distribution in the adult human diaphragm suggests approximately 55% slow, 21% fast oxidative and 24% fast glycolytic fibers (41, 42). The proportion of slow twitch fibers exceeds 60% in both the internal and external intercostal muscles, slightly higher than in the diaphragm (41, 42). This differential proportion of fiber types in the human respiratory muscles may also have resulted in a differential release of sTnI isoforms in serum following the volitional hyperpnea trial. The high percentage of slow twitch muscle fibers in the inspiratory muscles means that they may be preferentially damaged over fast twitch muscle fibers.

Limitations

Our study presents three limitations. Firstly, we encountered challenges in recruiting female participants to include their data in the current study. Despite our original study design including males and females, we were only able to recruit males only. Though a few females initially expressed their interest to participate, they perceived the volitional hyperpnea trial exhaustive and the esophageal catheter as too invasive, resulting in no female recruitment. This limits the generalizability of our findings. However, the purpose of this study was to elucidate the effects of volitional hyperpnea on biomarkers of respiratory muscle damage rather than to address potential sex-based differences. Therefore, future investigations should explore the impact of sex on changes in respiratory muscle damage biomarkers following volitional hyperpnea. Secondly, due to logistical constraints, serum samples were exclusively collected at 1, 24, and 48 hours after volitional hyperpnea. While these time points are suitable enough for detecting respiratory muscle damage, it is recommended that future research examine these biomarkers at additional intervals, both within and beyond the 24 h period. This approach would provide a more comprehensive understanding of the changes in these biomarkers in response to respiratory muscle damage. Finally, our sample size raises the possibility of type

458 II errors. This occurred due to challenges in recruitment, primarily due to the time
459 commitments, perceived invasiveness of testing, and restrictions on human testing due to
460 COVID-19.

461

462 ***Conclusion***

463 We investigated the response of a potential panel of serum biomarkers, consisting of CKM,
464 and fast and slow sTnI to detect the presence of respiratory muscle damage in response to
465 volitional hyperpnea and control trials undertaken on separate occasions in healthy young men.
466 The main finding was that only slow sTnI was higher at +24 h post volitional hyperpnea
467 compared to the same timepoint after the control trial. CKM and fast sTnI did not increase after
468 volitional hyperpnea compared with the control trial. These results suggest that respiratory
469 muscle damage may have been present at +24 h following volitional hyperpnea based upon our
470 serum slow sTnI findings, but not evidenced by our serum CKM and fast sTnI findings. Future
471 studies including more timepoints, different respiratory muscle exercise protocols and
472 examining the differences between sexes could provide additional insights into the utility of
473 blood biomarkers for identifying respiratory muscle damage.

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605 **Competing Interests:** The authors declare no conflict of interest.

606 **Author Contributions:** E.W., B.H., D.E.M., conceived and designed the experiments; M.I.,

607 E.B., D.E.M., performed the experiments; M.I. analyzed the data; M.I., D.E.M. wrote the paper.

608 E.B, E.W., B.H., D.E.M., reviewed the paper.

609 **Funding:** This research was funded by UniSQ International Research Scholarship, University

610 of Southern Queensland.

4.3. Links and implications

Studies 1 and 2 (chapters 3 and 4) aimed to assess respiratory muscle damage in healthy individuals by employing experimental overloading of the respiratory muscles. As concluded in these studies, the examination of these skeletal muscle damage biomarkers in a clinical population could be utilized to evaluate respiratory muscle damage and dysfunction in individuals with respiratory medical conditions.

Our subsequent approach was to assess respiratory muscle damage in specific respiratory conditions, such as COPD, or asthma. The prevalence of COVID-19 during my PhD was high. The available literature suggests that COVID-19 patients experience respiratory muscle damage, leading to reduced respiratory function and functional capacity often necessitating mechanical ventilation, which further increases susceptibility to muscle weakness. Therefore, we decided to investigate whether these biomarkers could be employed to assess respiratory muscle damage in COVID-19 patients weaned from mechanical ventilation and if inspiratory muscle training (IMT) might help mitigate this damage and improve respiratory function and functional capacity.

CHAPTER 5: PAPER 3 – The effects of inspiratory muscle training on biomarkers of muscle damage in recovered COVID-19 patients after weaning from mechanical ventilation

5.1. Introduction

Chapter 5 investigated the impact of inspiratory muscle training (IMT) on biomarkers associated with muscle damage in COVID-19 patients who had recovered after being weaned from mechanical ventilation. This chapter outlines that IMT can improve respiratory muscle function and functional capacity. IMT may play a role in restoring lost muscle coordination during mechanical ventilation by training the muscles to operate in a more synchronized manner, leading to more effective and efficient respiratory muscle function. The increased efficiency following training could potentially result in decreased concentrations of biomarkers associated with muscle damage. Prior to this study, the impact of IMT on reducing biomarkers of muscle damage in recovered COVID-19 patients post-weaning from mechanical ventilation had not been explored.

Participants were randomly assigned to either an IMT or control (CON) intervention for a four-week period. The IMT group engaged in 30 dynamic inspiratory efforts twice daily at 50% of their maximal inspiratory mouth pressure (P_{Imax}), while the CON group performed 60 inspiratory efforts at 10% of P_{Imax} daily. Four weeks of IMT resulted in decreased muscle damage biomarkers and increased respiratory function and grip strength in recovered COVID-19 patients after weaning from mechanical ventilation. This chapter proposes the incorporation of IMT into the management of COVID-19 patients, particularly those in intensive care, as it could contribute to their overall recovery.

5.2. Submitted paper

The effects of inspiratory muscle training in recovered COVID-19 patients

The effects of inspiratory muscle training on biomarkers of muscle damage in recovered COVID-19 patients after weaning from mechanical ventilation

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Running Head: The effects of inspiratory muscle training in recovered COVID-19 patients

Keywords: Biomarkers; Muscle damage; Inspiratory muscle training, COVID-19

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ABSTRACT

Background: COVID-19 patients experience respiratory muscle damage, leading to reduced respiratory function and functional capacity often requiring mechanical ventilation which further increases susceptibility to muscle weakness. Inspiratory muscle training (IMT) may help mitigate this damage and improve respiratory function and functional capacity.

Methods: We studied the effects of IMT on muscle damage biomarkers, respiratory function, and functional capacity in COVID-19 recovered young adults, successfully weaned from mechanical ventilation. Participants were randomly allocated to either an IMT (n=11) or control (CON; n=11) intervention for four weeks. The IMT group performed 30 dynamic inspiratory efforts twice daily, at 50% of their maximal inspiratory mouth pressure (P_{Imax}) while the CON group performed 60 inspiratory efforts at 10% of P_{Imax} daily. Serum was collected at baseline, week two, and week four to measure creatine kinase muscle-type (CKM), fast skeletal troponin-I (sTnI) and slow sTnI.

Results: Time x group interaction effects were observed for CKM and slow sTnI, but not for fast sTnI. Both were lower at two and four weeks for the IMT compared to the CON group, respectively. Time x group interaction effects were observed for forced expiratory volume in 1s, forced vital capacity, P_{Imax} and right- and left-hand grip strength. These were higher for the IMT compared to the CON group.

Conclusion: Four weeks of IMT decreased muscle damage biomarkers and increased respiratory function and grip strength in recovered COVID-19 patients after weaning from mechanical ventilation.

INTRODUCTION

Coronavirus disease (COVID-19) can reduce respiratory muscle function and functional capacity [1]. This can be through direct action of the virus (severe acute respiratory syndrome coronavirus-2) in the respiratory muscles causing muscle cell damage, inflammation and impaired muscle function [2, 3] and the resultant respiratory failure and increased work of breathing may necessitate mechanical ventilation [4]. Mechanical ventilation, while a life-saving intervention in patients with severe respiratory failure [5], may also lead to disuse atrophy, weakness and damage to the respiratory muscles [6, 7]. This can reduce respiratory function and functional capacity after the patient is weaned from mechanical ventilation [6, 7]. COVID-19 causes a distinct pattern of lung damage, characterized by diffuse alveolar damage and impaired gas exchange [2, 3]. This unique pathology leads to persistent respiratory impairments, including reduced lung compliance, decreased inspiratory muscle strength, and impaired gas exchange efficiency, even after patients have been weaned from mechanical ventilation [2, 3, 8].

As the respiratory muscles play an essential role in ventilation, damage to these muscles may have serious consequences and can lead to respiratory muscle dysfunction [9, 10]. This dysfunction can manifest as dyspnea and exercise intolerance through to ventilatory failure [10]. Previous studies have shown that the amount of respiratory muscle damage can be assessed by measuring concentrations of specific skeletal muscle damage biomarkers including creatine kinase muscle type (CKM), and slow and fast skeletal troponin I (sTnI) isoforms [11, 12].

Inspiratory muscle training (IMT) may help to restore muscle coordination lost during mechanical ventilation, by training the muscles to work together in a synchronized manner, resulting in more enhanced respiratory muscle function [13, 14]. This improved efficiency

following training may result in lower concentrations of biomarkers of muscle damage. There is some evidence that two weeks of IMT may improve pulmonary function, dyspnea, functional capacity and Quality of life (QoL) in recovered COVID-19 patients after weaning from mechanical ventilation [15]. However, a placebo or sham IMT group was not used in this study which weakens internal validity and risks participant bias. Whether IMT compared to a sham IMT control group could reduce biomarkers of muscle damage and improve respiratory function and functional capacity in recovered COVID-19 patients after weaning from mechanical ventilation is unknown.

Accordingly, we investigated the effects of IMT on biomarkers of muscle damage, respiratory function and functional capacity in recovered COVID-19 patients after weaning from mechanical ventilation. We hypothesized that IMT would reduce muscle damage and improve respiratory function and functional capacity compared to a control group using sham IMT.

METHODS

Participants

Twenty-two COVID-19 recovered young adults (Table 1), successfully weaned from mechanical ventilation within previous four weeks and having a COVID-19 negative report were recruited from University of Lahore Teaching Hospital, Lahore, Pakistan. The exclusion criteria were a body mass index (BMI) of <18.5 or >30 kg/m^2; current cigarette smoker; history of cardiopulmonary disease; or known disease and/or signs or symptoms of disease before COVID-19 who may be at a higher risk of an adverse event due to testing and/or IMT. All participants provided written, informed consent. All study procedures were approved by The

University of Lahore Teaching Hospital and by The University of Southern Queensland Human Research Ethics Committee (H21REA171), which adheres to the Declaration of Helsinki.

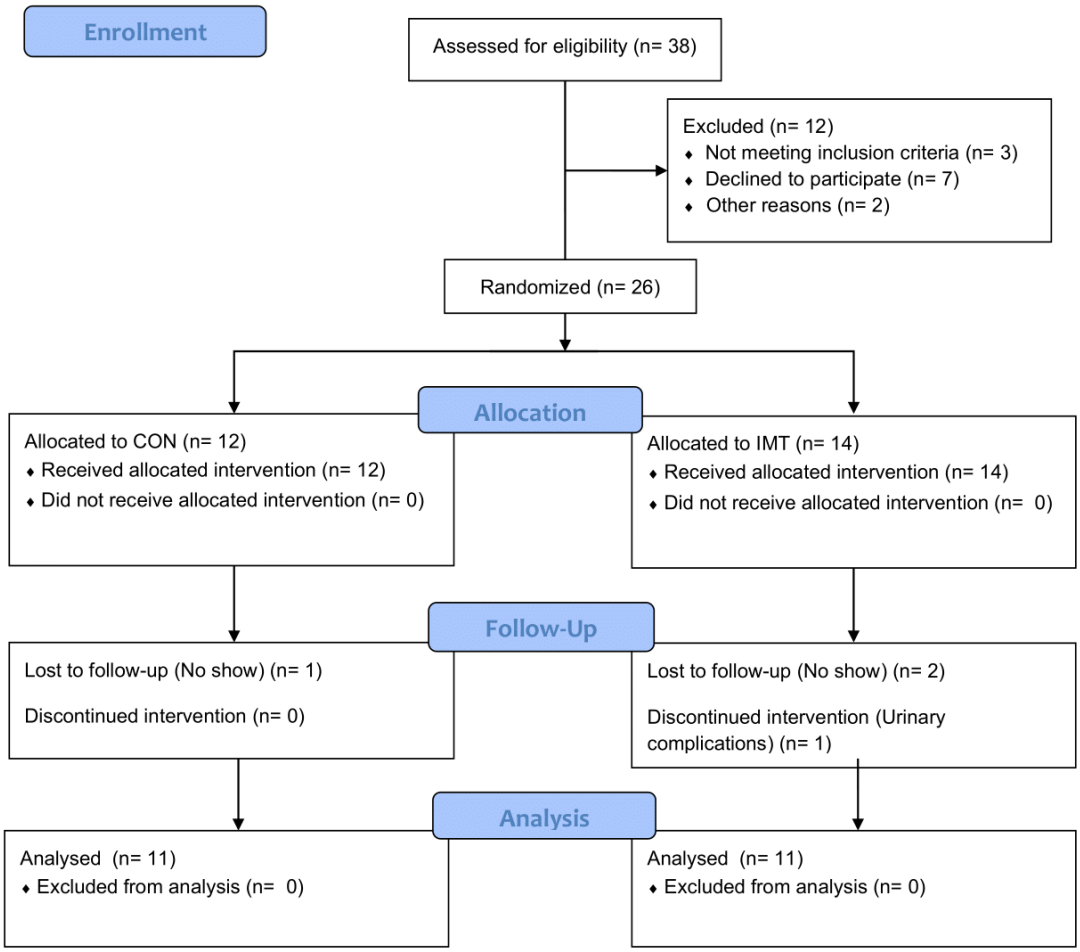

Figure 1. The CONSORT flow diagram of the study.

Experimental design

The study adopted a single-blind, randomized controlled design. Participants attended physiotherapy department of University of Lahore Teaching Hospital on four separate visits. The first was a screening visit where participants completed the Exercise and Sports Science

Australia Adult Pre-Exercise Screening System [16]. Subsequently, participants provided informed consent and height, body mass, pulmonary function, and maximal inspiratory mouth pressure (P_{Imax}) were assessed. Participants were then randomly, and equally divided into either an IMT or control (CON) group using Altman's minimization method (prioritizing BMI and sex) to ensure that the groups were balanced [17]. During the second visit, participants had a blood sample taken and undertook questionnaires for dyspnea using the MRC Dyspnea Scale [18] and QoL, and undertook a six-minute walk test (6MWT), grip strength test and sit-to-stand test and were familiarized with the IMT or CON interventions. During visits three and four, participants repeated same assessments as in visit two. Visits one and two were separated by 24 h and visits two, three and four by two weeks.

Anthropometrics

Participants' height was measured to the nearest 1 cm using a wall-mounted telescopic stadiometer (Seca220; Vogel & Halke, Hamburg, Germany) and body mass to the nearest 100 g using an electronic scale (Tanita Ultimate Scale 2000; Tanita, Tokyo, Japan).

Respiratory function

Participants performed standardized forced vital capacity (FVC) maneuvers using a spirometer (Spirolab; Medical International Research, New Berlin, WI, USA) according to published guidelines [19]. P_{Imax} was assessed using KH2 model IMT device (POWERbreathe International Ltd®, Warwickshire, UK) whilst participants were seated and wearing a nose clip and was initiated from residual volume. Repeat efforts were separated by 30 seconds and were performed until three serial measures differed by no more than 10% or 10 cmH_2O, whichever

was smallest [20]. The average of three highest values recorded were used for subsequent analysis.

Quality of life, handgrip strength, sit to stand and six-minute walk test

QoL was assessed utilizing the Euro Quality 5-Dimensions-3Levels (EQ-5D-3L) questionnaire [21, 22]. Handgrip strength was determined using hand dynamometry (Jamar Digital Plus; Lafayette Instruments, Lafayette, IN, USA) as previously described [23]. One-minute sit to stand testing was performed with a chair of standard height and without armrests. The participant was seated upright on the chair positioned against a wall. The participants were required to get up from this chair with the legs straight and sit back down whilst continuing the repetitions as fast as possible within 1 min [24]. Exercise performance was assessed by using a 6MWT according to published guidelines [25, 26].

Blood sampling and enzyme-linked immunosorbent assays

Twenty mL of venous blood was collected at each time point from an antecubital vein using a suitable method (either evacuated tube system or winged infusion) into serum separator tubes (BD Vacutainer® SST™ Tubes; Franklin Lakes, NJ, USA). The serum was separated, aliquoted and stored at -80°C until biochemical assays were performed. Enzyme-linked immunosorbent assays were performed for serum biomarkers using commercially available kits: Human CKM (Catalog No. RK01117 Abclonal, Woburn MA, USA); Fast sTnI (Catalog No. RK02421 Abclonal, Woburn MA, USA); and Slow sTnI (Catalog No. RK02420 Abclonal, Woburn MA, USA). ELISAs were performed by following manufacturers' instructions for each specific kit. To minimize the effect of inter-assay variation, markers from both the IMT and CON groups were measured using the same assay plate

Inspiratory muscle training and control interventions

The intervention lasted four weeks. The IMT and CON group were told they were a part of 'respiratory muscle strength training' OR 'respiratory muscle endurance training' intervention respectively. Both IMT and CON groups performed their respective training with an inspiratory pressure-threshold device (POWERbreathe Classic series 1st generation; Gaiam Ltd, Southam, UK). The IMT group performed 30 consecutive dynamic inspiratory efforts twice daily with initial training load set at 50% P_{Imax}. Thereafter, participants were instructed to periodically increase the load so that 30 maneuvers could only just be completed. Each inspiratory effort was to be initiated from residual volume, and participants were asked to strive to maximize tidal volume. This regimen is known to be effective in eliciting an adaptive response [27]. The CON group trained at a fixed intensity of 10% of P_{Imax} once per day for a total of 60 repetitions, five days/week. Both groups performed one supervised session at the start of intervention and during week two of intervention to monitor the appropriateness of their training technique and intensity. Compliance was assessed using a register of completed sessions.

Statistical analysis

Statistical analyses were performed using SPSS 25 for Windows (IBM, Chicago, IL, USA). An initial power calculation was performed on the basis of our previous work [11], showing that twelve participants would be required to demonstrate a 10% reduction in sTnI with an alpha of 0.05. Normality of the data was assessed by visual inspection of histograms. The data from both groups were analyzed using a two-way analysis of variance (ANOVA) procedure to determine the effects of 'time' (baseline, two and four weeks) and 'group' (IMT vs. CON). Following significant interaction effects, pairwise comparisons were made using the

Bonferroni method (adjustments for multiple comparisons). Statistical significance was set at $P < 0.05$. Results are presented as means ± SD.

RESULTS

Participant characteristics

Figure 1 shows the CONSORT participant flow diagram. Compliance with the interventions was good with 78 ± 4% and 91 ± 5% of the IMT and CON sessions completed, respectively. There were no differences in baseline participant characteristics between the groups (Table 1).

Table 1. Anthropometrics and respiratory function for the inspiratory muscle training (IMT) and control (CON) groups at baseline. Values are mean ± SD.

Variable	IMT (n = 11)	CON (n = 11)	P value
Age (years)	34 ± 6	32 ± 7	0.47
Sex (male/female)	8/3	7/4	
Height (cm)	171 ± 5.0	170 ± 5	0.76
Body mass (kg)	73.2 ± 6.6	75.8 ± 8.3	0.44
Body mass index (kg/m^2)	25.2 ± 1.5	26.2 ± 2.4	0.22
Days on mechanical ventilation	6.5 ± 3.4	8.1 ± 3.6	0.29
Baseline testing (days after weaning from mechanical ventilation)	19.5 ± 4.1	18.9 ± 5.3	0.80
FVC (% predicted)	79 ± 6	74 ± 6	0.31
FEV_1 (% predicted)	78 ± 5	75 ± 6	0.08
FEV_1/FVC (% predicted)	97 ± 3	98 ± 4	0.32
P_{Imax} (% predicted)	70 ± 13	72 ± 16	0.54

FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; P_{Imax}, maximal inspiratory mouth pressure. Predicted values for pulmonary volumes and capacities are from Quanjer et al. [28] and for P_{Imax} are from Wilson et al. [29].

Respiratory function and functional capacity

A two-way ANOVA revealed time x group interaction effects for forced expiratory volume in 1 s (FEV_1; $P = 0.007$), FVC ($P = 0.002$) and P_{Imax} ($P = 0.001$), but not for FEV_1/FVC ($P = 0.405$). FVC was higher for the IMT compared to the CON group at week two ($P = 0.022$) and

week four (P = 0.015) of the intervention. Both FEV_1 and P_{Imax} were higher (P < 0.05) for the IMT compared to CON group at week four of the intervention (Table 2).

A two-way ANOVA revealed time x group interaction effects for the distance covered during 6MWT (P = 0.001), sit to stands repetitions (P < 0.001) and right-hand (P < 0.001) and left-hand (P = 0.001) grip strength. There were no pairwise differences between the IMT and CON groups for the 6MWT distance and sit to stand repetitions. Right and left-hand grip strength was higher for the IMT compared to the CON group at week two (P = 0.05 P = 0.031) and week four (P = 0.013, P = 0.012) during the intervention.

Table 2. Respiratory function and functional capacity for the inspiratory muscle training (IMT) and control (CON) groups at baseline, week two and week four. Values are mean ± SD.

Variable	Group	Baseline	Week 2	Week 4
FEV_1 (L)	IMT	2.92 ± 0.45	3.06 ± 0.49	3.25 ± 0.53*
	CON	2.68 ± 0.32	2.77 ± 0.31	2.87 ± 0.31
FVC (L)	IMT	3.53 ± 0.54	3.81 ± 0.61*	4.04 ± 0.66*
	CON	3.15 ± 0.36	3.29 ± 0.35	3.45 ± 0.35
FEV_1/FVC	IMT	0.83 ± 0.03	0.80 ± 0.02	0.80 ± 0.01
	CON	0.85 ± 0.02	0.84 ± 0.02	0.83 ± 0.02
P_{Imax} (cmH_2O)	IMT	68 ± 6	75 ± 7	79 ± 7*
	CON	67 ± 6	71 ± 7	73 ± 6
Six-minute walk test distance (m)	IMT	333 ± 31	366 ± 38	378 ± 40
	CON	317 ± 55	331 ± 58	339 ± 60
Grip strength right hand (kg)	IMT	33 ± 6	39 ± 8*	45 ± 11*
	CON	30 ± 4	34 ± 4	36 ± 5
Grip strength left hand (kg)	IMT	31 ± 6	35 ± 8*	39 ± 8*
	CON	27 ± 3	30 ± 3	32 ± 3
Sit to stand (repetitions/min)	IMT	24 ± 3	27 ± 3	30 ± 4
	CON	24 ± 4	26 ± 4	28 ± 5

FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; P_{Imax}, maximal inspiratory mouth pressure. *Significantly different between IMT and CON (P < 0.05).

Quality of life and dyspnea

A two-way ANOVA revealed time x group interaction effects for QoL score on the visual analogue scale (P=0.001). QoL score on the visual analogue scale was higher for the IMT

223 compared to the CON group at week two (P=0.012) and week four (P<0.001) during the
224 intervention. None of the five dimensions of QoL questionnaire including self-care (P=0.190),
225 mobility (P=0.160), anxiety/depression (P=0.220), pain/discomfort (P=0.230) and usual
226 activity (P=0.390) showed significant time x group interaction effects. There were no time x
227 group interaction effects for dyspnea (P=0.418) (Table 3).

228 Table 3. Quality of life and dyspnea for the inspiratory muscle training (IMT) and control
229 (CON) groups at baseline, week two and week four. Values are mean ± SD.

Variable	Group	Baseline	Week 2	Week 4
Visual analog scale	IMT	47 ± 6	60 ± 8*	72 ± 7*
	CON	42 ± 7	51 ± 7	59 ± 7
Self-care	IMT	2.00 ± 0.45	1.18 ± 0.40	1.00 ± 0.00
	CON	2.27 ± 0.47	1.82 ± 0.40	1.36 ± 0.50
Mobility	IMT	2.00 ± 0.00	1.18 ± 0.40	1.09 ± 0.30
	CON	2.09 ± 0.30	1.64 ± 0.50	1.27 ± 0.47
Anxiety/depression	IMT	1.91 ± 0.54	1.55 ± 0.52	1.18 ± 0.40
	CON	2.18 ± 0.40	2.09 ± 0.30	1.82 ± 0.60
Pain/discomfort	IMT	1.73 ± 0.47	1.18 ± 0.40	1.09 ± 0.30
	CON	1.91 ± 0.30	1.64 ± 0.50	1.18 ± 0.40
Usual activity	IMT	2.00 ± 0.00	1.45 ± 0.52	1.09 ± 0.30
	CON	2.00 ± 0.00	1.64 ± 0.50	1.36 ± 0.50
Dyspnea	IMT	3.36 ± 0.50	2.18 ± 0.40	1.45 ± 0.52
	CON	3.64 ± 0.50	2.73 ± 0.65	2.00 ± 0.63

230 *Significantly different between IMT and CON ($P < 0.05$).

231

232 ***Muscle damage biomarkers***

233 A two-way ANOVA revealed time x group interaction effects for CKM ($P < 0.001$) and slow
234 sTnI ($P = 0.030$). Both were lower for the IMT compared to the CON group at week two ($P =$
235 0.001, $P = 0.002$) and week four ($P = 0.020$, $P = 0.001$) during the intervention. There were no
236 significant time x group interaction effects for fast sTnI ($P = 0.130$).

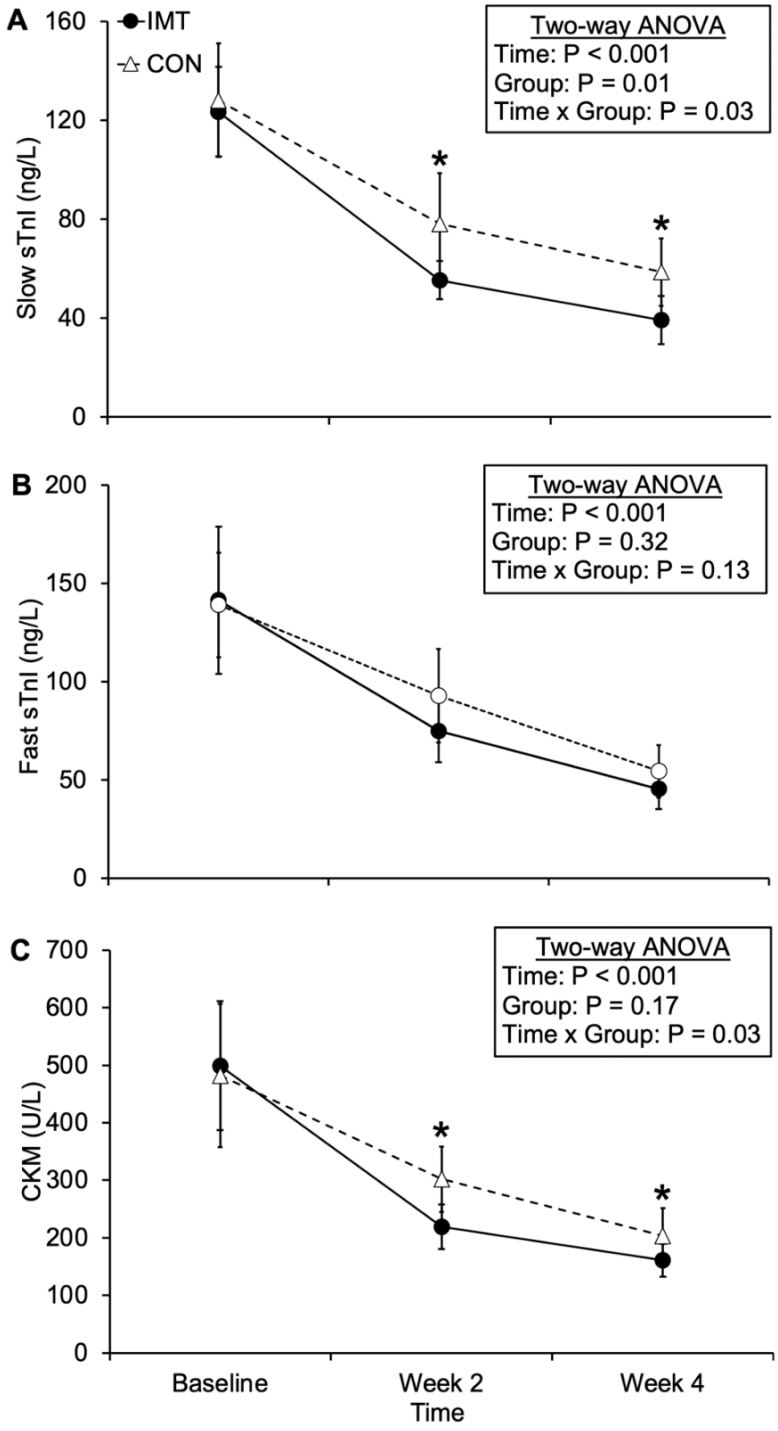

Figure 2. Serum slow skeletal troponin I (sTnI) (A), fast sTnI (B) and creatine kinase muscle type (CKM; C) for the inspiratory muscle training (IMT) and control (CON) groups at baseline, week two and week four. Values are means ± SD. *Significantly different between IMT and CON ($P < 0.05$).

DISCUSSION

Main findings

The main findings were that compared to the CON group, four weeks of IMT resulted in a reduction in muscle damage biomarkers sTnI and CKM. IMT improved pulmonary function and inspiratory muscle strength, measured through P_{Imax}. Four weeks of IMT also resulted in an increase in some components of physical functional capacity, as indicated by increased hand grip strength.

Muscle damage biomarkers

To our knowledge, we are the first to report the effects of IMT on muscle damage biomarkers including CKM, fast and slow sTnI in COVID-19 recovered patients, who had been weaned from mechanical ventilation. Previous studies [11, 12] have reported that these markers are more sensitive and specific biomarkers to evaluate respiratory muscle damage following conditions that elevate inspiratory muscle work or activity and causes respiratory muscle damage (i.e., inspiratory pressure threshold loading). Our results showed that the serum concentrations of CKM, fast and slow sTnI were higher at baseline in recovered COVID-19 patients who had been weaned from mechanical ventilation in the last four weeks than concentrations reported in healthy individuals [11, 12, 30]. COVID-19 patients may experience respiratory muscle damage either through direct viral invasion of the respiratory muscles causing muscle cell damage, inflammation and impaired muscle function [1-3]. The resultant respiratory failure and increased work of breathing may necessitate mechanical ventilation [4]. COVID-19 patients, weaned from mechanical ventilation, may have been experiencing respiratory muscle weakness and damage due to COVID-19 and the effects of mechanical ventilation.

Both the IMT and CON groups observed reduction in the concentrations of muscle damage biomarkers, but IMT resulted in a greater reduction in CKM and slow sTnI compared to the CON group. IMT has been found to be beneficial in the recovery from mechanical ventilation-induced respiratory muscle dysfunction by increasing respiratory muscle strength and endurance [31, 32]. IMT help to restore the muscle coordination lost during mechanical ventilation, by training the muscles to work together in a synchronized manner, resulting in more enhanced respiratory muscle function [13, 14]. This improved efficiency following training may have resulted in lower concentrations of biomarkers of muscle damage (CKM and slow sTnI). We did not find any changes in fast sTnI. This may indicate that our IMT protocol preferentially targeted slow fibers, whereas this protocol had less effect on fast fibers (i.e., fast sTnI). This could be explained on results presented by another study analyzing the structural adaptations and physiological outcomes of IMT in patients with chronic obstructive pulmonary disease (COPD) [33]. The proportion of type I fibers (~38%) and the size of type II fibers (21%) of the external intercostal muscles increase after IMT in COPD patients [33]. These findings establish that the external intercostal muscles of patients with COPD have the capacity to express differential structural remodeling after IMT.

Intensive care acquired muscle weakness is a common complication seen in critically ill patients, particularly those who have spent an extended period of time in the intensive care unit. While it is commonly associated with weakness in the respiratory muscles due to mechanical ventilation, it can also affect other muscle groups [34]. Critical illness myopathy leading to intensive care unit-acquired weakness is almost exclusively associated with severe atrophy, preferential loss of myosin, and altered muscle cell excitability. The measured biomarkers in both of the groups in our study could be influenced by skeletal muscles besides the respiratory muscles [34, 35].

Respiratory function

We observed an increase in FEV_1, FVC and P_{Imax}, but not FEV_1/FVC following IMT. FVC was higher for the IMT compared to the CON group at weeks two and four during the intervention. FEV_1 and P_{Imax} were higher for the IMT compared to the CON group at week four during the intervention. IMT may have improved lung function by improving the strength and endurance of the inspiratory muscles resulting in an increase in total lung capacity [36]. In healthy individuals, the principal limitation on total lung capacity is inspiratory muscle strength. Thus, if the inspiratory muscles are stronger, they can oppose the combined elastic recoil of the chest wall and lungs to a greater extent, and thereby a higher end-inspiratory lung volume can be achieved at full inflation (i.e., total lung capacity). Also, with a larger initial lung volume, there is slightly greater tethering of the airway, which would also facilitate a higher FEV_1, leading to a preserved FEV_1/FVC ratio [37, 38]. Our findings support a recent study that found an increase in P_{Imax}, FVC and FEV_1 following two weeks of IMT in COVID-19 patients following their weaning from mechanical ventilation [15].

Functional capacity

We observed time x group interaction effects for the distance covered during 6MWT, sit to stands repetitions and right- and left-hand grip strength. However, there were no pairwise differences between the IMT and CON groups for the 6MWT distance and sit to stand repetitions. Our results are not supported by Fernanda et. al [39], who showed that supervised pulmonary rehabilitation for eight weeks significantly increased the distance covered in 6MWT in severe COVID-19 patients weaned from mechanical ventilation. Our results suggest that as little as four weeks of IMT can improve grip strength, but this may not have been a long enough

duration to improve 6MWT distance and sit to stand repetitions. The 6MWT and sit to stand are more complex physical function tests compared to hand grip strength. IMT may improve the neural pathways involved in breathing and improve respiratory neuromuscular recruitment patterns [40]. This can lead to more efficient breathing mechanics, reduced wasted effort, and optimized energy expenditure during physical activities. The improved coordination and control of the respiratory muscles contribute to increased grip strength [41, 42].

Quality of life and dyspnea

We observed a higher QoL score for the VAS for the IMT compared to the CON group at week two and week four during the intervention. However, none of the five dimensions of QoL measured using the EQ-5D-3L changed significantly during intervention. Ahmed et. al [15] reported that total QoL VAS score in EQ-5D-3L questionnaire was reduced in COVID-19 patients after two weeks of IMT. However, this study did not report on the five dimensions of QoL nor did they use a sham IMT (control group). IMT may not directly address or significantly influence dimensions such as mobility, self-care, usual activities, pain/discomfort, and anxiety/depression, specifically in recovered COVID-19 patients after weaning from mechanical ventilation, who were critically ill previously. IMT may have a narrower scope of impact compared to interventions targeting these specific dimensions.

There were no time x group interaction effects for dyspnea and this was improved in both IMT and CON groups. Our dyspnea results are in accordance with a meta-analysis conducted by Figueiredo et. al [43] in which they analyzed 12 studies on the effects of IMT in COPD patients and reported that IMT did not change dyspnea. Reductions in dyspnea were though greater in studies that conducted IMT for eight weeks while studies with IMT durations of four to six

337 weeks reported no significant improvement in dyspnea [43]. Thus, our IMT duration may not be
338 long enough to elicit reductions in dyspnea.
339
340 ***Limitations***
341 Due to the restricted availability of suitable patients, a smaller number of female patients were
342 recruited, preventing the comparison of potential sex-related differences in outcome measures.
343 Furthermore, the study had a limited duration of supervision and follow-up observation. It is
344 essential to conduct randomized controlled trials with a larger patient cohort and an extended
345 supervision and observation period for comprehensive evaluation. Additionally, early initiation
346 of IMT during mechanical ventilation is recommended to prevent complications associated
347 with post COVID sequalae and mechanical ventilation.
348
349 ***Conclusion***
350 The aim of this study was to investigate the effects of IMT on biomarkers of muscle damage,
351 respiratory function and functional capacity in recovered COVID-19 patients after weaning
352 from mechanical ventilation. Our study concluded that four weeks of IMT results in reduction
353 of muscle damage biomarkers slow sTnI and CKM. IMT also improved pulmonary function,
354 inspiratory muscle strength and hand grip strength. The inclusion of IMT into the management
355 of COVID-19 patients, particularly for intensive care patients, could assist with their recovery.
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492 **Competing Interests:** The authors declare no conflict of interest.

493 **Author Contributions:** E.B., E.W., B.H., D.E.M., conceived and designed the experiments;

494 M.I., K.H, performed the experiments; M.I., D.E.M., analyzed the data; M.I., D.E.M. wrote the

495 paper. E.B., E.W., B.H., D.E.M., reviewed the paper.

496 **Funding:** This research was funded by UniSQ International Research Scholarship, University

497 of Southern Queensland.

5.3. Links and implications

This study suggested that the inclusion of IMT into the management of COVID-19 patients, particularly for intensive care patients, could assist with their recovery. Further clinical validation of these biomarkers in diverse clinical populations, such as individuals with COPD, asthma, or other respiratory diseases could also be undertaken. This step is crucial to ensuring that the biomarkers are applicable across a spectrum of respiratory conditions. Assessing whether the biomarkers hold diagnostic or prognostic value in diverse respiratory pathologies would assist in the management of respiratory conditions. These markers should be integrated with clinical signs and symptoms with other clinical information, medical history, and relevant physiological parameters. This holistic approach should aim to enhance the accuracy and clinical relevance of respiratory muscle damage assessment.

Future research could examine whether respiratory muscle biomarkers in response to ITL or VH are reduced following IMT. There is also a need to conduct studies that follow participants over an extended period of time to evaluate the sustainability of the observed benefits from IMT. This longitudinal approach can provide insights into whether the positive effects on muscle damage biomarkers persist over time. Optimal IMT protocols could also be research by systematically varying IMT parameters, such as intensity, frequency, and duration, to identify the most effective approach in reducing muscle damage. This could involve comparing different training regimens to determine the most efficient and sustainable strategy.

CHAPTER 6: DISCUSSION AND CONCLUSION

6.1. Overall findings

The overall aim of this research was to investigate whether damage to the respiratory muscles occurs after increased respiratory muscle work and to determine if serum biomarkers can be used to measure respiratory muscle damage. The following chapter will summarise the principal findings from each chapter, as well as the limitations and future directions of my research.

The aim of Study 1, presented in Chapter 3, was to investigate the response of a panel of biomarkers including fast and slow skeletal troponin I (sTnI), creatine kinase muscle type (CKM), fatty acid-binding protein 3 (FABP3), myosin light chain 3 (Myl3) and myoglobin in response to inspiratory pressure threshold loading (ITL) undertaken on separate occasions at 70% (high ITL) and ~0% of maximal inspiratory mouth pressure (P_{Imax}) (Sham ITL) in healthy young men. The main findings were that CKM (+1 h and +24 h), fast sTnI (+1 h) and slow sTnI (+48 h) post 70% ITL were higher compared to the same timepoints after Sham ITL. Furthermore, these same markers showed no changes following Sham ITL. There were no differences between FABP3, Myl3 and myoglobin between the high ITL and Sham ITL trials. These results suggest that respiratory muscle damage might be present 1, 24 and 48 h following 70% ITL based upon the serum CKM and fast sTnI and slow sTnI findings.

The aim of Study 2, presented in Chapter 4, was to investigate the effects of volitional hyperpnea (VH) on biomarkers of respiratory muscle damage including CKM, and fast and slow sTnI. The main finding was that only slow sTnI was higher at +24 h post VH compared to the same timepoint after control trial. CKM and fast sTnI did not increase after VH compared with the control trial. These results suggest that respiratory muscle damage may be present at +24 h following VH based upon the serum slow sTnI findings, but not evidenced by the serum CKM and fast sTnI findings.

The aim of Study 3, presented in Chapter 5, was to investigate the effects of IMT on biomarkers of muscle damage, respiratory function and functional capacity in recovered COVID-19 patients after weaning from mechanical ventilation. The main findings were that four weeks of inspiratory muscle training (IMT) resulted in a

decrease in respiratory muscle damage biomarkers sTnI and CKM. IMT improved pulmonary function and inspiratory muscle strength, measured through P_{Imax}. Four weeks of IMT also increased a component of physical functional capacity, as indicated by increased hand grip strength.

6.2. Respiratory muscle damage biomarkers after inspiratory pressure threshold loading

To my knowledge, this is the first study to report respiratory muscle damage biomarkers including fast and slow sTnI, CKM, myoglobin, FABP3 and Myl3 in response to ITL undertaken on separate occasions at 70% (high ITL) and ~0% of P_{Imax} (Sham ITL) in healthy young men.

Previously, Foster et al. [33] measured sTnI, a regulatory protein that plays an essential role in the contraction of skeletal and cardiac muscles [154, 155], following 60 min of ITL. They observed that fast sTnI increased at 1 h (+24%) and 3 days (+72%) post ITL. Slow sTnI was elevated by 24% 4 days post ITL. sTnI has the features of an ideal marker of skeletal muscle damage having absolute skeletal muscle specificity, broad diagnostic window that allows early (within 1-6 h after the onset) and late (after 24-48 h) diagnosis, and high sensitivity with a great magnitude of response [34]. Previous research demonstrates that fast sTnI, but not slow sTnI is elevated in the circulation immediately after strenuous exercise [156], even under extreme conditions [157]. Nonetheless, the application of fast and slow sTnI for studying and monitoring respiratory muscle damage is still scarce. Fast sTnI increased at 1 h post 70% ITL compared to Sham ITL. This finding is in accordance with Foster et al. [33] who reported that fast sTnI was also increased after 70% ITL. Similarly, slow sTnI concentrations were increased at 48 h after 70% ITL. These results are also in agreement with Foster et al. [33] who found that slow sTnI was elevated by 24% 4 days post ITL.

In contrast to the findings of our study, Foster et al. [33] did not find an increase in CK concentrations following 60 min of ITL at ~70% of P_{Imax} in young adults, which may be because they have measured total CK instead of the muscle type isoform CKM. Another factor that may explain why we observed changes in CKM is the relative

amount of loading performed in the ITL protocol. Foster et al. [33] utilized a breathing frequency of 10 breaths/min and a duty cycle of 0.3, whereas the present study utilized a breathing frequency of 15 breaths/min and a duty cycle of 0.5. This would ultimately result in greater loading of the respiratory muscles, and this could also explain the increase in CKM that were observed. Foster et al. [33] also did not use a Sham ITL condition.

Sorichter et al. [158] report that CK has a large within group variability and this makes it difficult to detect differences caused by damage in a relatively small group of muscles, such as the respiratory muscles, because the circulating blood volume can dilute its detection. The skeletal muscle isoform CKM was specifically targeted and this study found an increased CKM concentration at 1 and 24 h after 70% ITL as compared to Sham ITL. The results show that analyses of the CKM isoform in human serum can provide useful information on the extent and relative time course following an episode of respiratory muscle damage. CK shows significant within-group variability and falls within a normal reference range, making it challenging to distinguish differences caused by damage to relatively smaller muscle groups (i.e., the muscles of respiration). The circulating blood volume can dilute its detection. Foster et al. 2012 [33] proposed that sTnI could be a more sensitive marker for muscle damage. sTnI can detect respiratory muscle damage caused by ITL when other usual biomarkers may fail and offers an extra advantage of revealing a differential time course for slow and fast muscle fibers. Furthermore, sTnI has demonstrated the ability to identify muscle damage in patients with muscle disorders [159], triathletes [59], and following inspiratory muscle injury in rodents [116].

In this study we also evaluated other recommended biomarkers [16] that could be used to evaluate respiratory muscle damage following ITL. These included FABP3, a cytosolic lipid transport protein that is abundant in skeletal muscle and cardiac muscle [16]; Myl3, an essential light chain of the myosin molecule expressed predominantly in cardiac and skeletal muscles [60]; and myoglobin, an abundant haem-containing O_2 carrier expressed predominantly in type I skeletal fibers [160].

Following strenuous exercise, myoglobin is released as a result of degradation of protein structures within muscle [50]. After heavy exertion, myoglobin may increase

within 30 min [30], and remain increased for 5 days, probably due to low-grade inflammation [30]. In the present study, we observed that myoglobin increased at 48 h after 70% ITL relative to resting concentrations. However, this increase was similar to Sham ITL at the same time point. As such, myoglobin does not appear to be a suitable marker for respiratory muscle damage within the first 48 h following ITL. However, myoglobin may remain increased or continue to increase relative to Sham ITL after 48 h although we did not make any measurements past this time point to confirm.

Following damage to muscle tissue, the constituent subunits of myosin become dissociated, and Myl3 is released into the plasma. To date, efforts have focused on determining the utility of Myl3 as a biomarker of cardiomyocyte damage [60, 161]. Given its abundant expression in type I skeletal muscles, it has been noted that Myl3 may also be useful as a circulating surrogate for damage to type I myocytes [60]. Tonomura et al. [61] investigated the change in circulating Myl3 in response to a panel of cardiac and skeletal muscle toxicants and Myl3 accurately detected damage. In the present study, there was no change observed in Myl3 following ITL. This may be due to the loading of a small group of muscles (respiratory muscles), dilution of Myl3 in whole blood, or less intense damage to respiratory muscles as compared to toxicity-induced damage to whole-body skeletal muscles [61].

FABP3 has been shown to be expressed in skeletal muscles and expression is increased in response to physiological conditions that increase fatty acid demand/availability, such as testosterone, endurance training, and nutritional state [63]. Therefore, FABP3 has been proposed as a biomarker of both cardiac and skeletal muscle damage and has been previously shown to correlate with skeletal muscle degeneration [64]. We observed that FABP3 concentrations increased at 24 h post 70% ITL as compared to rest, but this increase was similar to the Sham ITL condition at the same time point. Our results are in accordance with Kanda et al. [26], who reported that after one-leg calf-raise exercise there was no increase in FABP3. FABP3 is increased following whole-body strenuous exercise damage [32, 162]. This finding may indicate that a large muscle mass needs to be activated [26] to observe an increase in FABP3. As the magnitude of the change in FAB3 is very small, this may also be due to type I error (false positive), possibly due to the small sample size.

Although from an experimental perspective, the durations of ITL and VH are reasonable and achievable in an experimental setting, the duration of increased loading of these physiological perturbations is a relatively short duration relative to patients experiencing acute or chronic ventilatory failure. Patients with acute or chronic ventilatory failure often face extended periods of respiratory compromise and physiological strain. This difference in duration is significant in understanding the clinical relevance and implications of various respiratory challenges and should be kept in consideration while using recommended biomarkers for clinical assessment of respiratory muscle damage.

6.3. Respiratory muscle damage biomarkers after volitional hyperpnea

To my knowledge, this is the first study to report previously investigated and recommended respiratory muscle damage biomarkers, CKM, fast sTnI and slow sTnI in healthy young men in response to VH. The VH challenge mimicked the breathing (tidal volume, breathing frequency and duty cycle) and diaphragm recruitment (transdiaphragmatic pressure) patterns achieved during high intensity exercise. We observed that slow sTnI was higher at +24 h post VH compared to the same timepoint after the control trial. These findings align with the outcomes of Study 1 where healthy young men performed 60 min of ITL at a resistance equivalent to 70% of their P_{Imax} and serum was collected at the same time points as the present study (+1 h, +24 h and +48 h) [163]. We found in that study that slow sTnI increased at +24 h and +48 h post ITL and is also in agreement with Foster et al. [33] who found that slow sTnI was elevated by 24% 4 days post ITL at a resistance equivalent to 70% of P_{Imax}. Our current study endorsed the conclusion mentioned by Foster et al. [33] that sTnI has superior sensitivity compared to other biomarkers or indices of respiratory muscle damage. ITL principally loaded the inspiratory muscles, whereas VH requires heightened recruitment of both inspiratory and expiratory muscles. Both primary and accessory muscles (of inspiration and expiration) underwent recruitment and overactivity during VH, thus we can suspect all of these respiratory muscles as potential sources of respiratory muscle damage biomarkers in serum.

We did not observe an increase in CKM and fast sTnI at any time point post VH compared to the control trial. This is in contrast to the previous findings using ITL [163], where we observed that CKM and fast sTnI can be utilized to evaluate respiratory muscle damage within 1 hour, whilst CKM can be utilized to evaluate respiratory muscle damage after 24 and 48 hours. The possible explanation for this finding is that the loading of the respiratory muscles during the VH protocol was not at a sufficient intensity to produce a significant amount of respiratory muscle damage as compared to ITL protocols used in our previous study [163]. Another possible explanation may be that CKM and fast sTnI are not sensitive enough to detect the phases of damage caused by VH as compared to slow sTnI. Further studies utilizing VH at differing intensities and durations to induce isolated respiratory muscle damage could clarify the relative sensitivities of these serum biomarkers to measure respiratory muscle damage.

6.4. Mechanisms of respiratory muscle damage

Respiratory muscle damage may occur during and following excessive loading, which exceeds the usual requirements of the muscle. Excessive loading can be categorized in two ways - overload or overactivity [85]. In respiratory disease, the respiratory muscles can experience both overload and overactivity because each breath may require a higher inspiratory muscle force and a higher breathing frequency (i.e., number of respiratory muscle contractions per minute). We inducted both ways to overload (Study 1) and to over-activate (Study 2) the respiratory muscles of healthy adult men to investigate respiratory muscle damage through recommended serum biomarkers.

In my first study, ITL was used to load the inspiratory muscles beyond the level of their regular activity [33, 130]. ITL requires significant quasi-isometric contractions of the inspiratory muscles (i.e., before the value opens); and this would probably augment muscle damage more than concentric contractions of inspiratory muscles. This makes ITL more ideal as a form of loading to induce damage than flow resistive loaded breathing. ITL induced respiratory muscle damage could be particular to just a few macromolecules of muscle tissue, or could result in small tears in the sarcolemma, z-disk, basal lamina, or supportive connective tissues, and damage to the cytoskeleton

and contractile elements [10-13]. In Study 1, we were able to detect significant changes in CKM and fast and slow sTnI in the blood of participants, indicating the presence of respiratory muscle damage following 70% ITL.

Overactivity can be defined as an increased work rate when the firing of the motor neuron is increased beyond its physiological levels or duty cycles [85]. Overactivity occurs during many endurance activities and in several disease conditions such as asthma or COPD [85]. The respiratory muscles can undergo both overload and overactivity in respiratory diseases due to the increased demand for inspiratory muscle force and frequency of breathing [85]. In Study 2, VH was used to induce a similar kind of overactivity of respiratory muscles, by voluntarily increasing breathing frequency and tidal volume.

This exceeded the respiratory muscles usual capacity, and this overactivity was aimed at causing damage to the muscles involved in breathing [33, 130]. The respiratory muscles may undergo mechanical strain, resulting in microtears in the muscle fibers and disruption of the sarcolemma and sarcomeres. ITL or VH induced respiratory muscle damage may also specifically affect some specific number of components in the muscle cells, or it could cause minor tears in various components such as the sarcolemma, z-disk, basal lamina, and surrounding connective tissues. Additionally, it can also lead to damage in the cytoskeleton and contractile elements [10-13]. This initial muscle damage initiates an inflammatory cascade characterized by the release of pro-inflammatory cytokines and chemokines. Neutrophils are recruited to the site of injury and become primed, displaying increased adhesion molecule expression and respiratory burst activity [164]. Concurrently, the inflammatory mediators may also induce vasodilation and heightened vascular permeability, facilitating the leakage of intracellular components, including sTnI, from damaged respiratory muscle cells into the circulation [10-13]. Elevated levels of circulating sTnI may serve as a biomarker for skeletal muscle damage. Following the initial inflammatory response, the damaged respiratory muscle fibers undergo a repair and remodeling processes, provided the injurious stimulus or activity has been removed, involving satellite cell activation, myoblast proliferation, and synthesis of new contractile proteins [165, 166]. However, severe or recurrent injury may lead to chronic inflammation, fibrosis, and functional impairment of the respiratory muscles [165, 166].

In addition to overactivity and overload that occur in conditions such as asthma and COPD, there can be other factors that contribute to exertion-induced respiratory muscle damage such as changes in operating length and prolonged overload. During both asthma and COPD, accentuation of airflow limitation may increase hyperinflation during an acute episode that will alter active range of motion relative to the usual length-tension relationship [167, 168]. The other major issue that may contribute to respiratory muscle injury during acute and chronic hyperinflation could be the relentless load imposed over hours or days with no rest [169].

An altered operating respiratory muscle length may be a contributor to ventilatory failure in acute exacerbations of asthma and COPD (due to increased hyperinflation) [170] as well as in mechanically ventilated patients (when positive end-expiratory pressure is imposed or discontinued) [171, 172]. Prolonged loading of the respiratory muscles, as seen in asthma and COPD exacerbations, or during mechanical ventilation [171], places continuous stress on these muscles due to the increased demand for breathing or the need for mechanical support. This sustained stress can result in changes to lung volume, such as hyperinflation, altering the operating length of the respiratory muscles. When the lungs become hyperinflated, it shifts the position of the diaphragm and other respiratory muscles, potentially impairing their ability to generate force efficiently. These alterations in operating length can compromise the respiratory muscles effectiveness in maintaining adequate ventilation. Consequently, this combination of prolonged loading and altered operating lengths may predispose individuals to ventilatory failure.

6.5. Effects of inspiratory muscle training on biomarkers of respiratory muscle damage in recovered COVID-19 patients after weaning from mechanical ventilation

To my knowledge, this is the first study to report the effects of IMT on muscle damage biomarkers including CKM, fast and slow sTnI in COVID-19 recovered patients, who had been weaned from mechanical ventilation. Previous studies [33, 163], including Study 1 and 2 in this thesis, have reported that these markers could be more sensitive and specific biomarkers to evaluate respiratory muscle damage in conditions that elevate respiratory muscle work. COVID-19 patients may experience respiratory

muscle damage either through direct viral invasion of the respiratory muscles causing muscle cell damage, inflammation and impaired muscle function, or due to acute hypoxic respiratory failure, pneumonia and/or acute respiratory distress syndrome [102-104]. The resultant respiratory failure and increased work of breathing may necessitate mechanical ventilation [173]. Along with providing positive pressure ventilation and increasing blood oxygenation, mechanical ventilation may also lead to disuse atrophy, weakness and damage of respiratory muscles, which can reduce respiratory function and functional capacity after the patient is weaned from mechanical ventilation [174, 175]. COVID-19 patients, weaned from mechanical ventilation, may have been experiencing respiratory muscle weakness and damage due to COVID-19 and the effects of mechanical ventilation. The results of Study 3 showed that the serum concentrations of CKM, fast and slow sTnI were higher at baseline in recovered COVID-19 patients who had been weaned from mechanical ventilation during last three weeks than concentrations reported in healthy individuals [163], depicting respiratory (or overall skeletal) muscle damage. It cannot be ascertained from this study whether this damage was from either the respiratory or peripheral muscles, or a combination of both. We could not compare baseline values with normal values from the same participants as blood samples were not taken before they had COVID-19. However, the baseline values obtained during their first visit (7-28 days after weaning from mechanical ventilation), were much higher than the normal/average values of these biomarkers in healthy adults [33, 163, 176], so we assumed that these participants were experiencing skeletal muscle damage and this possibly was from the respiratory muscles. It is also evident from the literature that respiratory muscle damage and weakness occur after weaning from mechanical ventilation [177, 178].

Another important aspect is that IMT can improve overall metabolic function and could enhance the body's ability to metabolize and clear metabolites [100, 179]. This could contribute to a more rapid clearance of muscle damage biomarkers from the bloodstream over time and IMT could have potentially accelerated their "disappearance" from the blood over time by metabolizing these biomarkers. This mechanism should clear all biomarkers, including CKM and fast sTnI, but just a significant "disappearance" was observed in slow sTnI, and not in the other two biomarkers, which supports our proposed mechanism. It is important to note that the

exact mechanisms by which IMT influences systemic metabolism, including the "disappearance" of muscle damage biomarkers, is not well understood. Research and further studies are needed to explore the potential metabolic effects of IMT comprehensively.

We observed that four weeks of IMT resulted in a reduction in CKM and slow sTnI compared to the control (CON) group. IMT has been found to be beneficial in the recovery from mechanical ventilation-induced respiratory muscle dysfunction by increasing respiratory muscle strength and endurance [180, 181]. IMT may help to restore the muscle coordination lost during mechanical ventilation, by training the muscles to work together in a synchronized manner, resulting in a more enhanced respiratory muscle function [152, 153]. This may have resulted in lower concentrations of biomarkers of muscle damage (CKM and slow sTnI). We did not find any changes in fast sTnI. This may indicate that the IMT protocol preferentially targeted slow fibers, whereas this protocol had no effect or little effect on fast fibers (i.e., fast sTnI). This could be explained by the results presented in another study analyzing the structural adaptations and physiological outcomes of IMT in patients with COPD [182]. The proportion of type I fibers (~38%) and the size of type II fibers (21%) of the external intercostal muscles increase after IMT in COPD patients [182]. These findings establish that the external intercostal muscles of patients with COPD have the capacity to express differential structural remodeling after IMT.

6.6. Effects of inspiratory muscle training on respiratory function and functional capacity in recovered COVID-19 patients after weaning from mechanical ventilation

Study 3 observed an increase in forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC) and P_{Imax}, but not FEV_1/FVC following IMT. FVC was higher for the IMT compared to the CON group at weeks two and four during the intervention. FEV_1 and P_{Imax} were higher for the IMT compared to the CON group at week four during the intervention. FVC may have improved in the IMT group due to greater inspiratory muscle strength and hence, a greater inspiratory vital capacity before the FVC maneuver. IMT may improve lung function and inspiratory muscle strength by improving the strength and endurance of the respiratory muscles [183]. IMT may also

help improve the coordination between the diaphragm, intercostal and other accessory muscles [184, 185]. This enhanced coordination may allow for a more efficient and synchronized muscle contractions during breathing. As a result, there could be better utilization of the available lung capacity and improved distribution of airflow within the lungs. Our findings support a recent study that found an increase in P_{Imax}, FVC and FEV_1 following two weeks of IMT in COVID-19 patients following their weaning from mechanical ventilation [112].

We observed time x group interaction effects for the distance covered during the six-minute walk test (6MWT), sit to stand repetitions and right- and left-hand grip strength. However, there were no pairwise differences between the IMT and CON groups for the 6MWT distance and sit to stand repetitions. The results suggest that as little as four weeks of IMT can improve grip strength, but this may not have been a long enough duration to improve 6MWT distance and sit to stand repetitions.

Enhancements in respiratory muscle strength and endurance may play a role in augmenting physical functional capacity through multiple physiological mechanisms [186]. IMT may improve the neural pathways involved in breathing and improve respiratory neuromuscular recruitment patterns [187]. This can lead to more efficient breathing mechanics, reduced wasted effort, and optimized energy expenditure during physical activities. The improved coordination and control of the respiratory muscles may therefore contribute to increased grip strength [188, 189]. As the diaphragm and intercostal muscles become stronger, the negative pressure generated during inspiration improves lung expansion, leading to increased O_2 intake [68]. This heightened O_2 availability contributes to enhanced aerobic energy production, optimizing the efficiency of oxygen utilization by working muscles [190]. Notably, the reduced perception of exertion during physical activities results from the improved ability of strengthened respiratory muscles to meet the respiratory demands of exercise [191]. Moreover, the coordination of respiratory muscles becomes better, promoting synchronized breathing patterns that are particularly beneficial for tasks requiring both upper and lower body movements, such as the sit to stand test [68, 192]. Collectively, these mechanisms highlight the integral role of respiratory muscle function in improving overall physical functional capacity or some of its components i.e., hand grip strength [68, 191].

Study 3 observed a higher quality of life (QoL) score for the visual analog scale for the IMT compared to the CON group at week two and week four during the intervention. However, none of the five dimensions of QoL measured using the EQ-5D-3L changed significantly during intervention. Ahmed et. al [112] reported that total QoL VAS score in EQ-5D-3L questionnaire was reduced in COVID-19 patients after two weeks of IMT. However, this study did not report on the five dimensions of QoL. IMT may not directly address or significantly influence dimensions such as mobility, self-care, usual activities, pain/discomfort, and anxiety/depression, specifically in recovered COVID-19 patients after weaning from mechanical ventilation, who were critically ill previously. IMT may have a narrower scope of impact compared to interventions targeting these specific dimensions.

There were no time x group interaction effects for dyspnea and this was improved in both IMT and CON groups. Our dyspnea results are in accordance with a meta-analysis conducted by Figueiredo et. al [193] in which they analyzed 12 studies on the effects of IMT in COPD patients and reported that IMT did not change dyspnea. Reductions in dyspnea were though greater in studies that conducted IMT for eight weeks while studies with IMT durations of four to six weeks reported no significant improvement in dyspnea [193].

6.7. Limitations

There were three limitations to our first two studies. Firstly, I encountered challenges in recruiting a sufficient number of female participants to include their data in these studies. As such, future research should factor in the effect of sex on changes in blood biomarkers following ITL and VH. Secondly, the sample sizes raise the possibility of type II errors. This occurred due to challenges in recruitment, primarily due to the time commitments, perceived invasiveness of testing, and restrictions on human testing due to COVID-19. Finally, due to logistical and budget constraints, blood samples were collected only at 1, 24 and 48 h post-ITL and post VH. While these time points are sufficient to identify the presence of respiratory muscle damage, future studies may analyze these same biomarkers at time points in between and beyond 48 h to better track the progression of these biomarkers in response to respiratory muscle damage. This approach would provide a more comprehensive understanding of the changes in these biomarkers in response to respiratory muscle damage.

The original aim of Study 3 was to investigate the effects of IMT in response to ITL or VH in healthy young adults. This was the natural progression from Study 1 and 2. However, when COVID-19 came to Australia I had to return to my home country in Pakistan to care for my family. Therefore, my supervisory team and I decided to change the aim of this study so I could complete my studies. As I had access to patients with COVID-19 in Pakistan through my employment as a physiotherapist, the aim of this study was changed to investigate the effects of IMT on biomarkers of respiratory muscle damage in recovered COVID-19 patients after weaning from mechanical ventilation. I believe that this is still in line with the overall topic of my thesis.

The main limitation of Study 3 is that it cannot be ascertained whether the muscle damage observed in patients was from the respiratory or peripheral muscles, or a combination of both. The original approach to this study was to expose the participants to a loading protocol (ITL or VH) and measure the amount of damage in response to this before and after IMT. However, for ethical reasons, this was not possible. Another limitation was the restricted availability of suitable patients and a smaller number of female patients were recruited, preventing the comparison of potential sex-related differences in outcome measures. The sample size raises the possibility of type II errors. I have undertaken a power calculation on the basis of the fast sTnI results at week four. IMT: 45.36 vs. CON: 54.36 ng/L based on a 12.48 ng/L standard deviation observed and a medium sized effect. It indicated that 30 participants would been needed for the IMT and CON groups to give 80% power to detect a significant ($P < 0.05$) difference. Furthermore, the study had a limited duration of supervision and follow-up observation. It is essential to conduct randomized controlled trials with a larger patient cohort and an extended supervision and observation period for comprehensive evaluation. Additionally, early initiation of IMT during mechanical ventilation is recommended to prevent complications associated with post COVID sequalae and mechanical ventilation.

6.8. Future directions

CKM, slow and fast sTnI could be used to assess respiratory muscle damage in experimental or respiratory conditions, and there are many future directions that still need to be explored.

1. Future studies should determine how CKM and sTnI specifically reflect respiratory muscle damage, considering variations in different populations and health conditions. This involves assessing whether these biomarkers might be influenced by factors other than respiratory muscle damage. Secondly, to investigate and identify other potential biomarkers that could complement CKM and sTnI in providing a more refined and comprehensive picture of respiratory muscle damage. This could involve analyzing markers associated with inflammation, oxidative stress [194], or other physiological processes related to muscle damage. Some of these biomarkers may be recommended to be used in routine testing along with CKM, slow and fast sTnI, while some of these (i.e., miRNAs [195], striated muscle-specific miRNAs [196]) could be used in future research to assess respiratory muscle damage.
2. Future research could be done to overcome the limitations of the three studies. Firstly, to conduct more frequent blood sampling during the acute phase of respiratory muscle stress or damage. This would involve collecting samples at shorter intervals to capture the early, intermediate, and late stages of the response, providing a detailed understanding of biomarker kinetics. To address the second limitation of the studies, research could be conducted to explore whether there are variations in the response of respiratory muscles to stress or damage between males and females. This could involve analyzing data separately for males and females to identify potential sex-specific patterns. From the available literature [197, 198], it appears that there are sex differences in various areas of respiratory exercise physiology and different responses could be observed if we compare males and females' levels of respiratory muscle damage and potential biomarkers. This research would also explore whether the identified biomarkers and assessment methods are applicable and relevant in assessing respiratory muscle damage in the pediatric and older population as the respiratory system undergoes various anatomical, physiological and immunological changes with age [199] and different age specific responses could be observed in different populations.

3. Future research could assess the response of respiratory muscle damage biomarkers following ITL or VH of different intensities and durations. It could be hypothesized that the duration of respiratory muscle work may be more important rather than the intensity based on studies 1 and 2.
4. Further clinical validation of these biomarkers in diverse clinical populations, such as individuals with COPD, asthma, or other respiratory diseases. This step is crucial to ensuring that the biomarkers are applicable across a spectrum of respiratory conditions. Assessing whether the biomarkers hold diagnostic or prognostic value in diverse respiratory pathologies would assist in the management of respiratory conditions. These markers could be integrated with clinical signs and symptoms with other clinical information, medical history, and relevant physiological parameters. This holistic approach should aim to enhance the accuracy and clinical relevance of respiratory muscle damage assessment.
5. Exertion-induced muscle damage can be reversible (as is expected after the experimental techniques used in this thesis (i.e., ITL or VH) and is an integral component of the training response of muscle, especially in young, healthy adults. However, at a certain magnitude, damage/injury may not be reversible and can result in connective tissue replacement, i.e., in certain muscular dystrophies or chronic inflammatory myopathies. There is a need to consider these conditions distinctly and future studies should explore the applicability of recommended biomarkers in such conditions separately.
6. With respect to Study 3, future research could examine whether respiratory muscle biomarkers in response to ITL or VH are reduced following IMT. There is also a need to conduct studies that follow participants over an extended period to evaluate the sustainability of the observed benefits from IMT. This longitudinal approach can provide insights into whether the positive effects on muscle damage biomarkers persist over time. Optimal IMT protocols could also be research by systematically varying IMT parameters, such as intensity, frequency, and duration, to identify the most effective approach in reducing muscle damage. This could involve comparing different training regimens to determine the most efficient and sustainable strategy.

By addressing these aspects in future research, researchers can enhance the understanding of respiratory muscle damage, refine assessment tools, and contribute

valuable knowledge to the fields of exercise physiology, respiratory medicine, and rehabilitation.

6.9. Summary

Chapter 3 demonstrated that CKM (1 h and 24 h), fast sTnI (1 h) and slow sTnI (48 h) post 70% ITL were higher compared to the same timepoints after sham ITL. These results suggest that respiratory muscle damage was present at 1, 24 and 48 h following 70% ITL, and CKM and fast sTnI could be used to assess respiratory muscle damage immediately (1 h), while CKM and slow sTnI could be used to assess respiratory muscle damage 24 and 48 h following conditions, such as ITL, that elevate respiratory muscle work. Chapter 4 demonstrated that only slow sTnI was higher at +24 h post VH compared to the same timepoint after the control trial. CKM and fast sTnI did not increase after VH compared with the control trial. These results suggest that respiratory muscle damage may be present at +24 h following VH and delayed release of sTnI could be employed to assess respiratory muscle damage 24 h after conditions, such as VH, that elevate the activity of respiratory muscles. Chapter 5 reported the effects of IMT on these biomarkers of respiratory muscle damage, respiratory function and functional capacity in recovered COVID-19 patients after weaning from mechanical ventilation. The main findings were that four weeks of IMT resulted in a reduction in respiratory muscle damage biomarkers slow sTnI and CKM. IMT also improved pulmonary function, inspiratory muscle strength and functional capacity. The inclusion of IMT into the management of COVID-19 patients, particularly for intensive care patients, could assist with their recovery.

In summary, this thesis highlights that serum CKM and fast sTnI could be used to assess respiratory muscle damage immediately, while CKM and slow sTnI could be used to assess respiratory muscle damage at later stages following conditions or diseases that elevate respiratory muscle work or activity and cause respiratory muscle damage. The increase in these biomarkers depends upon the experimental setup or the respiratory disease, as the level of muscle damage varies based on these factors. Instead, the thesis suggests that it is more useful to use a combination of these biomarkers at various timepoints, along with considering other clinical signs and symptoms, along with medical history, or experimental protocol details. This approach could provide a more accurate and comprehensive assessment of respiratory muscle

damage. The specificity of these biomarkers for different time points needs further investigation in other protocols or diseases that cause elevated inspiratory muscle work. Moreover, examining these biomarkers in diverse clinical populations with various respiratory medical conditions would help in determining their specificity and sensitivity. This thesis also suggests the inclusion of IMT into the management of COVID-19 patients, particularly for intensive care patients, could assist with their recovery.

REFERENCES

Note that the references presented here are for Chapters 2 (literature review) and 6 (discussion) only. The references used for Chapters 3, 4 and 5 are included in the references' sections of the papers. The references for these chapters follow the formatting guidelines requested by each of the journals in which they have been submitted to or accepted.

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